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From the Department of Experimental Medicine, Pharmacia AB, Uppsala, Sweden

Protein Concentration in Interstitial and Lymphatic Fluids from the Subcutaneous Tissue

By

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Received 24 February 1976

Abstract

Rutili, G. and K. E. Arfors. *Protein concentration in interstitial and lymphatic fluids from the subcutaneous tissue.* Acta physiol. scand. 1977. 99. 1–8.

The protein content of interstitial fluid and lymph from the same tissue was determined, fluid samples of nanoliter volumes being taken from the subcutaneous tissue of rabbits by micropuncture. In the same area lymph was collected from lymphatic vessels of 30–100 μm. An electrophoretic technique in polyacrylamide gels in glass capillaries was used for the analysis of proteins. The gels were stained and then scanned on a microscope equipped with a moving table and photomultiplier. The area under each protein band was calculated from the recorded densitograms. The method required only a few nanolitres of fluid for analysis. Using solutions of known protein concentrations the relationship between the amount of protein and the area under the densitogram band was investigated. This relationship was found to be linear making it possible to quantify the protein content of both interstitial fluid and lymph samples. The interstitial fluid/plasma concentration ratios for albumin, transferrin, globulins and total proteins were 0.42, [illegible], [illegible] and 0.37. The albumin/globulin ratio in interstitial fluid was found to be 1.60 of the plasma value although the values obtained for lymph were similar to those found for interstitial fluid. The similarity between the protein concentration of interstitial fluid and lymph indicated that the endothelial lining of the terminal lymphatics did not restrict the passage of macromolecules into the lymphatics.

Key words: Interstitial fluid, lymph, proteins, subcutaneous tissue, permeability

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According to the classical Starling-Landis concept, capillary fluid balance is maintained by dynamic interaction between hydrostatic and osmotic forces acting across the capillary wall. These forces are considered to be of almost equal magnitude, therefore cancelling each other out. Efforts have been made during recent years to measure the numerical values of the forces, but Wiederhielm (1971) has pointed out that the literature on capillary fluid balance still contains a number of apparently conflicting results.

One of these conflicts results from the fact that while direct measurements of hydrostatic pressure (Richardson and Zweifach 1970) have been made possible by sensitive micropressure devices (Intaglietta *et al.* 1970), the bulk of our information about interstitial fluid osmotic pressure is derived from measurement of lymph protein concentration. Experimental evidence that tissue fluid and lymph are identical in their protein composition is still lacking. Moreover in recent reports (Casley-Smith and Bolton 1973, Casley-Smith

1976) it has been suggested that as a result of lymphatic compression, lymph is ultra-filtr through the endothelium thus increasing the protein concentration of the lymph as c pared to the interstitial fluid concentration

Direct measurements of interstitial fluid protein concentration are difficult since o minute amounts of fluid are available by sampling from connective tissue, and adequ analytical methods for protein determination in such small volumes have not been availa until now *Indirect estimates of interstitial fluid protein concentration have been made* determination of the partition of protein between plasma and the tissue space (Bensoc *al* 1955 Walker *et al* 1960 Studer and Potchen 1968) These measurements, howev yield results which are intrinsically an expression of the average body or organ protein c centration thus making comparison with lymph protein concentration difficult.

In the present investigation a technique making it possible to analyse the protein cont of samples in the nanolitre range has been used to determine the protein concentration both interstitial fluid and lymph from the same tissue The method consists of micropu ture for the collection of lymph and tissue fluid and an electrophoretic technique for t quantitative analysis of proteins in nanolitre samples of fluid.

Materials and methods

Prot in dete mination

The protein content of interstitial fluid, lymph and plasma was determined by the disc-electrophore method of Ornestein (1964) and Davis (1964). A micro-scale modification, and the practical details, ha been extensively described by Smeds (1969). Certain further modifications were, however made before t study *In brief the electrophoresis was run in small glass capillaries of 3 µl vol me (Drummond Sci. C* Broomall, Penn U S A) with i d 200 µm containing spacer and a run i g polyacrylamide gel. The co centration used was 5% in the spacer nd 20% i the running gel. These concentrations were double the used by Smeds (1969) and resulted in ncreased resolution of the d fferent proteins.

Ammoniumperoxydisulf t was used instead of riboflavin because it leads t more uniform cross-linki in the polyacrylamide. The pulsed voltage over the gel was kept at 175 V with pulse rate of 500 H a duty-cycle of 25 After the run the gel was pushed out of the capillary and placed in solution of 20 sulfosalicylic acid fo 15 min. Treatment of the gel with sulfosalicylic acid was performed to mak the bin ing of Commassie Blue to the protn more table. After staining for 15 min 0 2 solution of Coo massie Blue the gel was washed several times a solution of acetic acid, methanol nd distilled wat (1 5 10) and stored f r 1 h the same soluti n.

A photomicrograph of the gel is shown in F g 1 A total of 15 prot bands were stained but only a bumin, transferrin and α_2 macroglobulin identified The optical density of the stained protein discs w measured *l LEITZ ORTHOLUX microscope, f tted with photomult plier* (EMI KNOTT Ty 6094 A, Knott Elektronik, Munich, West Germany) A logarithmic I tegrator Recorder (V tatron N V Dieren, Holland) gave recordings of protei extinction. Fo measurement the gel was placed in a slit (5 0.4 mm) made i brass plat fixed o to plexiglass plate. A cover glass was placed over the slit t kee the gel wet during measurement. The gel was scanned at 6 mm/min using powered microscope table A adjustable slit i the l ght path of the microscope perm tted measurement of specified width of the gel A yellow filter (LEITZ 510) was also placed i the light beam. A densitogram is shown n F g 2.

To make quantitative measurements of the different proteins in the gel, the relationship between th area under the curve and the amount of protein w s investigated with sol tions of standard huma serum proteins (Boeringwerk AG West Germa y) of known concentrations. The volumes of tandard solution used varied between 1 1 and 15.0 nl.

Sampling of interstitial fluid and lymph

The experiments were performed on 12 rabbits. The details of the micropuncture technique used for collection of interstitial fluid have been described previously (Rutili and Arfors 1976).



Fig 1 Photomicrograph of polyacrylamide gel electrophoresis of an interstitial fluid sample after staining with Coomassie Blue The volume of the sample was 8 4 nl.

In summary after careful dissection of the epidermal layer of the rabbit's hindlegs, the subcutaneous tissue was exposed In the dissected area the microcirculation was readily visible and lymphatics of 30 to 100 μm diameter could occasionally be observed. Lymphatic vessels smaller than 30 μm and terminal sacs could not be identified Using micromanipulator small oil-filled glass pipette with tip diameter of 8-10 μm was inserted into the tissue and fluid collected by slight aspiration produced by withdrawing 4–5 ml of air with syringe connected to the pipette holder The sample was placed in teflon cup filled with paraffin oil so that the droplet assumed spherical shape and its volume could be calculated by measurements of its diameter The whole sample was then removed and placed onto the polyacrylamide gel After electrophoresis the gel was analysed for its protein content. Since total of 20 samples could be run in parallel the analysis was carried simultaneously with 4 lymph samples, 4 interstitial samples, 4 plasma and 8 standards for each rabbit. The volume of the standard protein solution was determined in the same way as for the interstitial fluid samples

Results

The relationships between the amounts of albumin, transferrin, globulins and total proteins, and the areas under absorption curves obtained from standard solutions were investigated.

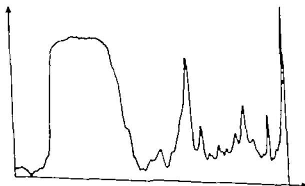


Fig 2 Densitometric recording of the separation pattern of an interstitial fluid sample The gel was scanned on microscope equipped with slowly moving table and photomultiplier for measurement of the optical density.

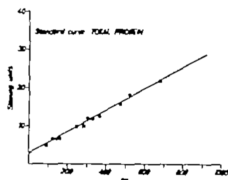


Fig. 3

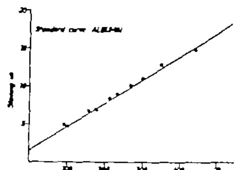


Fig. 4

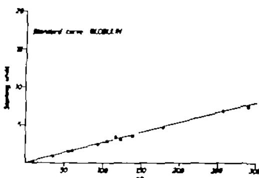


Fig. 5

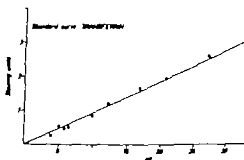


Fig. 6

Fig. 3 4 5 6. Relationship between the amount of Total Proteins, Albumin, Total Globul ns, Transfen and the amount of Commassie Blue stai as measured from the optical density of the stained protein bam The amou t of stain is expressed as Staining Units. (For more details see text)

The results are shown in Fig. 3 4 5 and 6. The area under each curve is expressed in stai ing units, which are calculated for each protein fraction using the formula S U I l/s I I is the number of integration impulses, s is the reading given by a standard reference sign for the chart recorder which varies with the sensitivity adjustment of the recorder and E the fixed value of the standard signal (0 2)

An almost linear relation for the different proteins was found within the range used i this investigation and the coefficient of variation of the analytical procedure was calculate and found to be less than 12%. The protein concentrations of lymph (L) and interstitu fluid (I F) measured in the subcutaneous tissues of rabbits are given in Table I

TABLE I Plasma, lymph and terstitial fluid protein concentration (g/100 ml) of the bcutaneous tissu of rabbits The values re mean ± S.D of 1 nimals.

	Tot. protein	Albumin	Globulins	Transferrin
Plasma	6 63	3 62	2.68	0.27
S.D	0.58	0 56	0.40	0.03
Lymph	2.66	1 81	0 82	0.11
S.D	0 40	0.37	0.20	0 0.
I t. fluid	2 45	1 60	0.86	0.11
S D	0.41	0.30	0 19	0 02

TABLE II. Interstitial fluid and lymph/plasma proteins. Concentration ratio of rabbit subcutaneous tissue.

	Tot. protein	Albumin	Globulins	Transferrin	A/G
Int. fluid	0.37	0.42	0.32	0.42	1.60
S D	0.06	0.08	0.07	0.09	0.22
Lymph	0.38	0.46	0.30	0.41	1.70
S D	0.06	0.08	0.09	0.08	0.50

No significant difference between lymph and interstitial fluids was observed within the standard error of the method.

In Table II the protein concentrations of I.F. and lymph are compared with the respective plasma values.

The values for the albumin/globulin (A/G) of Table II are also expressed as ratios of the plasma values. No difference was found between I.F. and lymph.

The similarity between interstitial fluid and lymph is shown more directly in Table III. The values for albumin, globulins and transferrin determinations of the single experiment are given as a mean ratio between interstitial fluid and lymph concentration.

To check the reliability of the micro-electrophoretic method for quantitative determination of proteins, plasma samples from 6 rabbits were analysed for their protein content by the macro-zone electrophoretic standard method. The albumin/globulin ratios (A/G) obtained with the micro-zone electrophoretic method (I) were 1.40, 1.44, 1.25, 1.56, 0.81, 0.98 and those obtained with the micro-disk electrophoretic method (II) were 1.41, 1.50, 1.15, 1.52, 0.92, 1.00 respectively. The ratio I/II was 0.99 ± 0.06.

Discussion

The electrophoretic method used in this investigation for both qualitative and quantitative analysis has provided a means of measuring the protein concentrations of body fluid samples of nanolitre volumes. The relatively high coefficient of variation of the method (12) should be considered in relation to the small volumes of the samples used for protein analysis (1.1-15 nl). Comparative analysis of the same samples by an independent method showed no significant difference between the methods and confirmed the validity of the micro-disc electrophoretic technique. As the relationship between staining units and protein concentration was linear quantitative determinations of protein concentrations in interstitial fluid, lymph and plasma were possible. The validity of the micropuncture sampling technique for the collection of tissue fluid has been discussed elsewhere (Rutili and Ar

TABLE III. Albumin, globulins and transferrin interstitial fluid to lymph concentration ratio. The values are mean ± S.D. of twelve rabbits.

Albumin	Globulins	Transferrin
0.91 ± 0.12	1.09 ± 0.21	1.0_ ± 0.17

(a) The value of each single rabbit was obtained from 2 to 4 interstitial and lymph fluid samples.

fors, 1976) The overall findings of the study are consistent with the view that the collected are of interstitial fluid The time required for dissection of the epidermal layer and collection of fluid samples (≃5 min) is relatively short, and changes due to trauma and inflammation are minimal

The concentration of total protein in the interstitial fluid and lymph from the rabbit subcutaneous tissue was found to be lower than the concentration in plasma (1 3). The values found are in agreement with those previously reported for the same tissue and species Haljamäe and Freden (1970) found an interstitial fluid to plasma protein of 0.32 and Courtice (1960) a lymph to plasma protein ratio of 0 41

Using nylon wicks to collect interstitial fluid, Aukland and Fadnes (1973) found an interstitial fluid to plasma protein ratio of 0 56 in rats which is somewhat higher than the value found in this investigation Our lower values may be due to a species difference. In studies where interstitial fluid collected using wicks were directly compared with interstitial fluid collected by micropuncture (Rutili and Arfors, to be published) no significant difference was found between the two methods.

Of the 15–16 different proteins stained, only the albumin, transferrin and total globulin concentrations were determined separately α_2-Macroglobulin could not be quantified accurately because of its low concentration. The concentrations of these protein fractions in the interstitial fluid and in the lymph were identical (Table I) The similarity in composition between the two fluids suggests that the passage of macromolecules from the tissues to the lymphatics is not restricted by the endothelial lining of the terminal lymphatics. The morphological explanation of this no-restriction concept was given by Leak and Burke (1968) and by Cliff and Nicoll (1970). In electron microscopic studies these workers have found "flaps and gaps" to be present in the endothelial layer of the terminal lymphatics permitting the passage of quite large molecules. Moreover they found anchoring filaments connecting the small lymphatics to the surrounding tissue, representing the mechanism responsible for opening the lymphatic capillaries in conditions of increased tissue pressure. The lack of a concentration gradient between the tissue fluid and the lymph fluid also indicates that the net transport of macromolecules between the two compartments is, at steady-state a convective rather than a diffusive process If this is a valid argument it will follow that the concentration of proteins in the tissue is, within their volume of distribution evenly distributed. The capillary wall will thus represent the site of selectivity to molecular transport and the steady-state lymph/plasma ratio a measure of its permeability Garlick and Renkin (1970) arrived at a similar conclusion as a result of kinetic studies of the passage of different macromolecules from blood to lymph. The hypothesis that lymph protein concentration is higher than interstitial fluid concentration (Casley-Smith and Bolton 1973 Casley Smith 1976) providing the driving force for osmotic flow from interstitial fluid into lymphatic capillaries, is not supported by the present results. The idea that net transport of proteins from interstitial space into lymph by an osmotic flow occurring as a result of a concentration gradient of the same proteins, has already been shown to be incompatible with the physical laws of osmotic flow (Michel 1974)

The albumin/globulin (A/G) ratio in tissue fluid and lymph was found to be higher than the A/G ratio of plasma by a factor of 1 6–1 7 The absolute values of the A/G ratio

in interstitial fluid and lymph are not given in the results because of the large variation found in the A/G ratio of plasma between different rabbits (Table III). The mean A/G ratio of plasma was, however 1.35 compared to a mean A/G ratio in tissue fluid of 2.20. A similar tissue fluid value (2.67) was found in the same tissue by Haljamäe and Freden (1970). The higher A/G ratio of interstitial fluid relatively to plasma indicates a higher permeability of vessel walls to albumin than to globulins. The transport of proteins across the microcirculation is, according to Lassen *et al.* (1974) a unidirectional process. The functional morphological structure in the capillary walls through which this process takes place has been described by Grotte (1956). From studies of the transcapillary passage of dextran of increasing molecular size, he suggested the presence in the capillary wall of pores of 35–45 Å radius, excluding molecules of the size of albumin, and fewer large pores of 120–350 Å of radius through which the filtration of plasma could occur.

The effective filtration area of porous membrane available for molecule is function of the effective molecular radius (R_{EM}) and of the pore radius. Equations for the calculation of the effective filtration area have been formulated by Renkin (1954). Using these equations and assuming a R_{EM} for albumin of 35 Å and a R_{EM} for globulins of 50 Å, the A/G ratio found in these experiments may be explained in terms of filtration through pores of 250–300 Å diameter in the capillary wall.

An alternative to the large pore hypothesis is vesicular transport. Endothelial vesicles were firstly described by Palade (1953) after electron microscopic observation of the fine structure of the capillary wall. Electron dense tracers such as Ferritin (R_{EM} 110 Å) or Gold (R_{EM} 100 Å) injected into the circulation were found to be localized within the vesicles (Karnovsky 1968) suggesting their participation in transporting material across the capillary wall. Vesicular transport is subject to steric restriction (Garlick and Renkin 1970). Assuming a vesicle radius of 250 Å (Bruns and Palade 1968, Casley Smith 1968) globulins will be restricted by factor 1.2–1.3 relative to albumin. This explains only in part the measured A/G ratio of 1.6–1.7 and the difference can only be explained by assuming an additional transport of albumin through the small pore system.

The identical C_i / C_p ratio of albumin and transferrin (molecular weight 70 000 and 88 000 respectively) is of interest considering the difference in the electrical charge of the two molecules (2 1 at pH 8.6, isoelectric points 4.9 and 5.9 respectively) and suggests that the permeability of vessels to proteins is a function of molecular size.

We are grateful to Mr. Ove Forsberg for the skilful technical assistance in the electrophoretic analyses of this study.

References

AUKLAND, K. and H. O. FADNES, Protein concentration of interstitial fluid collected from rat skin by wick method. *Acta physiol. scand.* 1973. *88*. 350–358.

BENSON, J. A., K. S. KIM and J. L. BOLLMAN, Extravascular diffusion of proteins. *Amer. J. Physiol.* 1955. *182*. 217–220.

BRUNS, R. and G. PALADE, Studies on blood capillaries. *J. cell. Biol.* 1968. *37*. 244–299.

CASLEY-SMITH, J. R., The dimensions and numbers of small vesicles in blood and lymphatic endothelium and in mesothelium. *J. Anat.* 1968. *103*. 202–203. (Abstract.)

CASLEY-SMITH, J. R. The functioning and interrelationship of blood capillaries and lymphatics. *Experientia (Basel)* 1976. *32*. 1–12.

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The albumin/globulin (A/G) ratio in tissue fluid and lymph was found to be higher than the A/G ratio of plasma by a factor of 1 6–1 7 The absolute values of the A/G ratio

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Pineal Serotonin Metabolism in Non-Innervated Perinatal Glands before and after Intraocular Maturation
Supersensitivity of Adrenoceptors that have never been Innervated

By

MARIA BÄCKSTRÖM, LARS OLSON and ÅKE SEIGER

Received 23 March 1976

Abstract

BÄCKSTRÖM, M., L. OLSON and Å. SEIGER. *Pineal serotonin metabolism in non-innervated perinatal glands before and after intraocular maturation: Supersensitivity of adrenoceptors that have never been innervated.* Acta physiol. scand. 1977 99 9–18.

Transplantations were made of fetal pineal glands (crown rump length, CRL, 19–30 mm) or pineal glands from adult male rats to the anterior chamber of the eye of the rat. Studies were performed with regard to the importance of the age of the donor animal (and thereby the degree of maturation and innervation of the gland to be transplanted) for the possible development of denervation supersensitivity. The transplants were cultured in medium containing ^{14}C-serotonin. Increased production of ^{14}C N-acetylserotonin (NAcS) was used as the main criterion for β-adrenergic stimulation. 4 experimental groups were obtained by transplanting fetal or adult pineals to intact or sympathetically denervated eyes. In all 4 groups β_1 stimulation (KWD 2033 10^{-4} M) increased ^{14}C NAcS formation. The response to β-stimulation was significantly higher in denervated fetal pineal transplants than in innervated fetal transplants and thus demonstrating β-receptor supersensitivity. It was concluded that a) the ability to respond to β-adrenoceptor stimulation with increased ^{14}C-NAcS formation develops between the 18th and 20th day of gestation, b) transplants derived from fetal as well as from adult rats can respond to β-adrenergic stimulation, c) this sensitivity also develops in ocular transplants that at the time of transplantation lacked the capacity to increase their ^{14}C-NAcS formation in response to treatment with β-agonist, d) denervation supersensitivity occurs in fetal transplants that became mature in sympathetically denervated eyes.

Key words: Pineal, transplants, serotonin, β-adrenergic, denervation supersensitivity, reinnervation.

The rat pineal gland metabolizes serotonin to N-acetylserotonin (NAcS) and melatonin (MEL) or 5-hydroxyindoleacetic acid (5-HIAA) (Wurtman *et al.* 1968). The activity of N-acetyltransferase (NAT), N-acetylating serotonin to NAcS (Weissbach *et al.* 1960), is controlled by β-adrenergic stimulation of the pineal noradrenoceptor mediated through increased intracellular production of cyclic adenosine 3',5'-monophosphate (cAMP) (Klein, Berg and Weller 1970). In our previous study homologous intraocular transplants from newborn rats were assayed 2.5–3.0 months following transplantation and were found to have

CASLEY-SMITH J R. and T BOLTON The presence of large colloidal osmotic pressures across large pores. *Microvasc R s.* 1973 5 213 216.

CLIFF W J and P A NICOLL, Structure and function of lymphatic vessels of the bat s wing. *Quart J. exp Physiol* 1970 55 112 121

COURTICE, F C., Determination of protein fractions in small samples of plasma and of lymph in the rabbit *Aust J exp Biol med Sci.* 1960. 38 395–402.

DAVIS, B J Disc-electrophoresis. II Method and pplication to human serum proteins. *Ann. A Y Acad Sci* 1964 *121* 404–426.

GARLICK, D G and E. M RENKIN, Transport of large molecules from plasma to interstitial fluid and lymph in dogs. *Amer J Physiol* 1970. *219* 1595 1605.

GROTTE, G Passage of dextran molecules across the blood lymph barrier *A ta chir scand.* 1956. Suppl *211* 1–84

HALJAMÄE, H and H FREDEN, Comparative analysis of the protein content of local subcutaneous tissue fluid and plasma *Microvasc Res.* 1970. 2. 163–171

INTAGLIETTA, M R F PAWULA and W R TOMPKINS, Pressure measurements in the mammalian microvasculature. *Microvasc R s.* 1970. *16* 294–300.

KARNOWSKY M J The ultrastructural basis of capillary exchange. *J gen Physiol* 1968. 52 64–95.

LASSEN, N A H H PARVING and N ROSSING, Filtration as the main mechanism of overall transcapillary protein escape from the plasma *Microvasc Res.* 1974 7 I–I Editorial.

LEAK, L. V and J F BURKE, Ultrastructural studies on the lymphatic anchoring filaments. *J cell Biol* 1968. *36* 129–149

MICHEL, C. C. The transport of solutes by osmotic flow with particular reference to the transport of proteins across the venous fenestrae of visceral capillaries. *Microvasc Res.* 1974. 8 1.. 1 4

ORNSTEIN L., Disc-electrophoresis. I Background and theories. *Ann. N Y Acad. Sci* 1964 *121* 321 349

PALADE, G F Fine structure of blood capillaries. *J appl Ph siol* 1953 *24* 1424 (Abstract)

RENKIN, E. M Filtration diffusion, and molecula sieving through porous cellulose membrane *J gen Physiol* 1954 *38* 225–243

RICHARDSON, D R and B W ZWEIFACH, Pressure relationship on the macro- and micro-circulation of the mesentery *Microvasc R s.* 1970 474–488.

RUTILI, G and K. E. ARFORS, Fluorescein labelled dextran measurement in interstitial fluid in studies of macromolecular permeability *Microvasc Res.* 1976. I press.

SMITH, S., Analysis of thyroid proteins by means of micro disc-electrophoresis. *J Chromatograph* 1969 *44* 148 160

STUDER, R. and J POTCHEN, The radioisotopic assessment of regional microvascular permeability to macromolecules *Microvasc R* 1971 3 35–48

WALKER, W G R S. ROSS nd J D S. HAMMOND, Study of the relationship between plasma volume nd tra scapillary protein excha ge usi g [131]I labelled lbumin a d [131]I-labelled globulins *Circulat Re* 1960. 8 1028–1040.

WIEDERHIELM, C. A. *Dynamics of capillary fluid xchange* Proc. Int. U ion of Phy iol Sciences. XXV I t. Congress, *M rch 1971 Vol. III* p. 261 6..

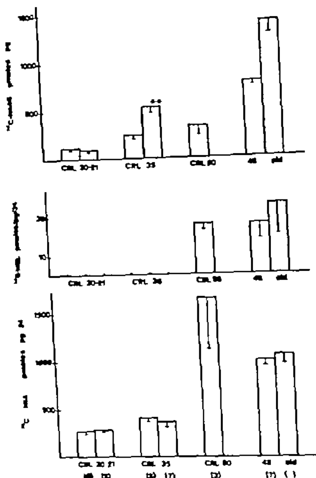


Fig. 1. Production of ^{14}C-serotonin derivatives by cultured immature rat pineal glands. ^{14}C NAcS = ^{14}C N-Acetylserotonin, ^{14}C MEL = ^{14}C Melatonin, ^{14}C 5-HIAA = ^{14}C 5-Hydroxyindoleacetic acid. CRL values in crown-rump length in mm of the animals used. Empty and shaded bars indicate mean values of ^{14}C-labelled metabolite formation in unstimulated controls or KWD 2033 (10^{-4} M) stimulated glands respectively, measured as picomoles of metabolite formed per pineal gland per 24 h culture period. Standard error of the mean is indicated by vertical line in bar. The number of pineal glands cultured per group presented in brackets below bars displaying ^{14}C 5-HIAA formation. ** designate groups that differ significantly (p 0.01) from their respective control groups.

h. Following the preincubation period KWD 2033 (10^{-4} M) was added to some of the vessels as stated in Table I and Fig. 2. All pineals were cultured according to the method developed by Trowell (1959), modified by Rous (1965) and first elaborated for pineal organ culture by Klein and Weller (1970). The glands were each cultured in 0.6 ml of BGJ_b-medium, Fitton-Jackson modification (Grand Island Biological Company). Bovine serum albumin (1 mg/ml), ascorbic acid (0.1 mg/ml) and 1-glutamine (0.3 mg/ml) were added on the day of the expt. The medium contained 0.25 mM ^{14}C 5-hydroxytryptamine (serotonin) creatinine sulphate (Radiochemical Centre Amersham), specific activity 54 mCi/mmol, diluted with unlabelled serotonin (Fluka AG) to 4 mCi/mmol. No correction was feasible for the decrease in ^{14}C-serotonin specific activity due to endogenous production of serotonin from 1-tryptophan contained in the culture medium. The culture chamber was continuously exposed to 95 O_2 and 5% CO_2 at 37°C. All tissues were

matured and to have become innervated by sympathetic nerves. They were responsive to external lighting conditions with pronounced increases of NAT (20 1) and hydroxyindole-0-methyl transferase (HIOMT) activities during dark hours as compared to light activities (Bäckström, Olson and Seiger 1976) Similar findings have recently been reported by Moore (1975) Sympathetic denervation of the host eye eliminated the dark induced rise in HIOMT activity and greatly attenuated NAT enzyme variation (2 1). Cholinergic iris nerves have been shown to readily reinnervate eye chamber transplants that receive cholinergic fibers in their normal locus (Tsukahara, Jacobowitz and Laties 1972). The constant presence of cholinergic nerves in all host irides did not modify the dependence on an intact adrenergic innervation

The persistent low amplitude NAT variation in denervated eye chamber pineal transplants was statistically significant. One explanation for this variation could be the development of supersensitivity to circulating catecholamines in the transplants subsequent to denervation (Cannon and Rosenblueth 1949 Trendelenburg 1966) Development of denervation supersensitivity to β-adrenoceptor stimulation in rat pineal glands has been described (Deguchi and Axelrod, 1972, 1973 Bäckström and Wetterberg 1973 Strada and Weiss 1974).

It was desired to investigate whether the degree of maturation and sympathetic innervation of the grafted pineals could influence the eventual status of indole metabolism. Would receptor supersensitivity in response to β-adrenoceptor stimulation develop in a pineal gland transplanted into a sympathetically denervated eye, at a stage prior to the sympathetic fiber development in the gland? Thus, would the receptor become supersensitive without ever having been contacted by its appropriate nerve supply?

Materials and methods

Animals. Albino rats (Sprague-Dawley) were fed food pellets and water *ad libitum* and kept one (pregna t rats) or five t cage. Lights were between 06 and 18 h. Pregnant rats, used for direct organ culture of fetal pineals, were decapitated between 10 nd 16 h. Uteri were quickly dissected out and kept on ice while preparations of tissues were performed Pineal glands from fetal rats w th CRL 20–21 mm, 50 mm and from 48 h ld rats were each taken from one litter Pineal gland from fetal rats with CRL 35 mm were taken from two litters. The fetal or newly born rats were decapitated and using a dissection microscope the pineal glands were freed from adherent tissue. Pineal glands were stored in ice cold medium for 1 2 min before being troduced into the culture chamber Normal control rats were 300–350 g males, housed for 14 days on the same light dark cycle. Host animals were sacrificed by decapitation between 9.30 and 12 30 h. The eyes were immediately removed and the transplants kept on ice during the dissection. Simultaneously processed *in situ* pineal glands were dissected out and stored in ice-cold ncubation medium. All tissues were placed in the incubation chamber within pproximately 3 min after decapitation.

Transplantations. Pineal glands from fetal rats with CRL 19–30 mm (18–20 days of gestation) and from dult male rats (150–200 g) were bilaterally transplanted into the anterior hamber of the eye, adult recipient male rats. Pineals were rapidly dissected out from the donors, decapsulated nd troduced int the nterior eye chamber thro gh a slit i the cornea w th a modified Pasteur pipette according to Olson and Malmfors (1970). All recipient animals (totally 33 rats) underwent unilateral sympathetic denerv tion by extirpation of either the left or the right superio cervical ganglion few days before transpla tation. The cholinergic innervation of the recipient iris was in all cases left intact. The taking and development of the transplants were followed *in vivo* by repeated inspections through the cornea using tereoscopic microscope under light ether anesthesia. The postoperative time ranged from 3 to 4 months.

Organ culture Pineal glands from fetal rats and newly born rats were preincubated for 1 2 h before the β_1-adrenoceptor agonist KWD 2033 (10^{-4} M) (Carlsson *et al.* 1975) was added as indicated in F g. 1 Pineal transplants and *in sit* pineal glands from experimental and normal rats were preincubated for ~4

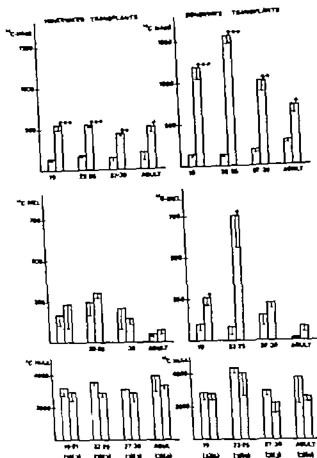


Fig. 2. Production of ^{14}C-serotonin derivatives by cultured innervated and denervated rat pineal transplants derived from donor rats of known ages. The left part of the figure shows metabolite formation in innervated transplants, the right shows denervated transplants. ^{14}C-NAcS: ^{14}C-N-Acetylserotonin; ^{14}C MEL: ^{14}C Melatonin; ^{14}C-HIAA: ^{14}C 5-Hydroxyindoleacetic acid. Numbers below bars give crown-rump length (CRL) in mm of transplant donor animals. Empty and shaded bars illustrate mean values of ^{14}C-labelled metabolite formation in unstimulated controls or KWD 2033 (10^{-4} M) stimulated transplants respectively, measured as picomoles of metabolites formed per transplant per 24 h culture period. Standard error of the mean is indicated by vertical line in bar. The number of pineal transplants cultured per group is presented within brackets below the bars which display ^{14}C 5-HIAA formation. and designate groups that differ significantly from their respective control groups ($p < 0.05$, p 0.01 and p 0.001 respectively).

The production of ^{14}C 5-HIAA also rose with age, reaching 25–30% of adult levels in 48 h old pineals (Fig. 1). KWD 2033 (10^{-4} M) did not affect the formation of ^{14}C 5-HIAA.

Normal mature and transplanted glands. In vitro pineal glands from normal rats and experimental rats (carrying bilateral eye chamber transplants) produced amounts of ^{14}C NAcS, ^{14}C MEL and ^{14}C 5-HIAA that did not differ statistically from each other when controls or KWD 2033 (10^{-4} M) treated groups were compared. In both types of glands KWD 2033 increased the production of ^{14}C NAcS (5.4- and 4.0-fold respectively) and ^{14}C

TABLE I Production of ^{14}C-serotonin derivatives by cultured pineal glands and transplants.

Pineal types	Treatment		Metabolites in medium		
			N Acetyl 5-HT	Melatonin	5 HIAA
I situ normal	Co trol	(4)	15 ±32	338±67	3 930±155
	KWD 2033	(7)	828±146	656±61	2 556±473
In situ experimental	Control	(6)	167±30	294±45	3 120±168
	KWD 2033	(4)	664±215	688±138	2 904±528
All innervated transplants	Control	(16)	159±21	170±41	3 430±344
	KWD 2033	(13)	512±26	182±41	3 078±189
All denervated transplants	Control	(14)	186±27	87±22	3 406±409
	KWD 2033	(16)	1 128±96	332±79	2 945±377

Data are expressed as mean ± standard error of the mean of picomoles ^{14}C-labelled metabol te formed pe pineal per 24 h culture period, preceded by 1 h preincubation N Acetyl-5-HT = N-Acetylserotonin 5 HIAA = 5-Hydroxyindoleacetic acid N mbers in brackets designate the number of pineal glands or trans plants cultured per group. KWD 2033 concentration was in all cases 10^{-4} M and ndicate group that differ significa tly i amou ts of ^{14}C-metabolites formed as compared t their respecti e control groups $p<0\ 05$ $p<0\ 01$ a d $p<0.001$ respectively

cultured for 24 h following add tion of β-adrenoceptor agonist (Bäckström and Wetterberg 1973). Media were collected and frozen at −20°C. The ^{14}C-labelled serotonin metabolites ere separated by th n-la er chromatography as described by Klein and Notides (1969). Radioactivity was eluted in 95 ethanol or 0.1 M HCl Insta-Gel® (Packard Instrument International S A.) or Aquasol (New England Nucle) were added and radioactivity determined by liquid scintillation (Packard Tri-Carb 3380, supplied w th an Absolute Activity Analyzer 544).

Doses of KWD 2033 were given as the final concentration of the base in culture medium

Statistical analyses were perf rmed by application of Student s two-tailed t test for comparison of means.

Results

In vivo observations All pineals survived transplantation into the anterior chamber of the eye regardless of the stage of development at operation. They became rapidly vascularized from the iris of the host eye The most immature pineals (CRL 19–21 mm) were approximately 1 1 mm at operation and grew *in oculo* to a final size of approximately 2 1 5 mm. Pineals from CRL stages 22–25 mm and 27–30 mm were initially somewhat larger and reached the same final size as the most immature pineals. Adult pineals were approximately 2 1 5 mm already at transplantation, and did not change size *in oculo* Denervated transplants were visibly smaller than innervated transplants regardless of donor age

Immature pineals Pineal glands from the most immature fetal rats (CRL 20–21 mm) formed ^{14}C NAcS The production increased with maturity (Fig. 1). After addition of KWD 2033 (10^{-4} M) the pineal glands from fetal rats with CRL 35 mm and 2 day old rats significantly increased their production of ^{14}C NAcS (2 and 1 8 times respectively) as compared to controls ($p<0\ 01$ and $p<0\ 001$ respectively)

There was *no* production of ^{14}C MEL in pineal glands from fetal rats with CRL 20–21 mm or CRL 35 mm either untreated or following stimulation with KWD 2033 (10 M) (Fig. 1). Pineal glands from fetal rats with CRL 50 mm and from 2 day old rats produced minimal amounts of ^{14}C MEL and this production was not altered by addition of drug.

Ontogenetic development of serotonin metabolism. In earlier works pineals from fetal rats have been reported to lack responsive adenyl cyclase which constitutes part of the proposed pineal postjunctional adrenergic receptor (Weiss 1971). However pineal glands from fetal rats with CRL 35 mm (about 20–21st day of gestation) are found to double their ^{14}C NAcS formation in response to addition of the directly acting β-adrenoceptor agonist KWD 2033. This response was not observed in pineal glands from the most immature fetal rats with CRL 20–21 mm (about 17–18th day of gestation). NAT enzyme activity has been detected as early as 4 days before partus. The initially high values of NAT decreased markedly around 7 days of age (Ellison, Weller and Klein 1972). Similarly in comparison with mature pineal glands a higher unstimulated level of ^{14}C NAcS was formed around and 2 days subsequent to birth as seen in our present cultures. Our findings are compatible with the report of Weiss and Strada (1972) revealing a large increase of rat pineal phosphodiesterase activity between day 8 and 16 post partum. An increased breakdown of cAMP would lower the stimulation of postjunctional biochemical events required for maintaining NAT activity. HIOMT activity is hardly detectable during the first 10–12 days post partum in the rat pineal but then rises sharply to reach adult values at 34–50 days of age (Zweig and Snyder 1968, Snyder 1968, Klein and Lines 1969). A diurnal variation of HIOMT activity has first been detected in 39 day old rats (Klein and Lines 1969). Thus, the present absence or extremely low values of MEL production in very young rats were in good agreement with earlier reports. Low levels of MAO enzyme activity which is responsible for the deamination of serotonin to 5-HIAA, have been observed in pineals of newborn rats (Zweig and Snyder 1968, Snyder 1968). In the present organ cultures ^{14}C 5-HIAA formation was noted when pineals were explanted 4 days before birth, at a stage when pineals are not yet sympathetically innervated (Machado 1971). The ^{14}C 5-HIAA was thus formed extraneuronally probably within pinealocytes.

Transplantations. Normal adult *in situ* pineal glands were cultured in the same experiment as pineal transplants and *in situ* pineal glands from experimental animals (carrying bilateral ocular transplants and submitted to unilateral superior cervical ganglionectomy). Pineal glands from normal adult rats did not differ in their production of ^{14}C-labelled NAcS, MEL or 5-HIAA as compared to the *in situ* pineal glands from experimental rats either unstimulated or β-stimulated with KWD 2033. This shows that the one remaining superior cervical ganglion supplied the *in situ* pineal glands with sufficient sympathetic nerves to ensure a normal adrenergic response. It also follows that the eye chamber transplants did not influence the activity of the *in situ* pineal gland to any significant extent.

Denervated transplants from fetal donors gave a higher increase of ^{14}C NAcS production following β-stimulation with KWD 2033 than did innervated β-stimulated transplants taken from rats of corresponding ages indicating the development of supersensitivity to aminergic stimulation. This phenomenon was less pronounced in grafted pineals from adult donors although they were the only transplants which were actually denervated. Innervated grafts from all age groups responded to β-stimulation with an increased ^{14}C NAcS production of about the same magnitude as normal pineal glands. ^{14}C MEL formation in transplants was lower than in the *in situ* pineal glands both in denervated and innervated transplants. It was shown by Bäckström, Olson and Seiger (1976) that denervation of transplants caused

MEL (1 9- and 2.3-fold respectively) ($p < 0\ 05$) but did not alter [14]C 5-HIAA formati-- (Table I)

When all non-stimulated transplants from the different donor groups were compiled th was no difference between those that were sympathetically innervated and those that w denervated in the formation of [14]C-labelled NAcS, MEL or 5-HIAA per transplant (Table β-stimulation by KWD 2033 (10^{-6} M) caused a 2.7 times larger increase in [14]C NAcS pr duction in denervated transplants than in innervated transplants ($p < 0\ 001$) In the c nervated group KWD 2033 increased [14]C NAcS formation 6.1 times (Table I) This la [14]C NAcS increase, induced by KWD 2033 was significantly higher than in the *in s* glands of the experimental rats. *KWD 2033 (10^{-6} M) also caused a 3 8-fold increase in t* formation of [14]C MEL in denervated transplants ($p < 0\ 01$). In innervated transplants the was no increase in [14]C MEL formation following KWD 2033 addition (Table I).

When innervated and denervated transplants were analyzed with respect to the matur of the donor animal it was found that the three different fetal groups were approximate equivalent (Fig. 2) while transplants obtained from adult donors were found to increa their [14]C NAcS formation less in response to β-stimulation. They also had lost much c their [14]C MEL synthesizing capacity [14]C 5-HIAA formation in transplants was not affecte by denervation or drug treatment (Table I and Fig. 2)

Discussion

In the denervated eye chamber pineal transplants a low amplitude variation of NAT en zyme activity persisted (Bäckström, Olson and Seiger 1976) This might have been due to development of denervation supersensitivity of the transplants, which might have re sponded to circulating catecholamines. The eye chamber transplantation technique also offered a possibility to study such a phenomenon in very immature fetal pineal glands tha had not been innervated by the time of operation and where ganglionectomy would have posed technical difficulties. It may thus be possible that these fetal tissues develop an ad renergic receptor supersensitivity without ever having been in contact with their appropriate nerves.

Culture medium content of the 3 [14]C-serotonin metabolites NAcS, MEL and 5-HIAA (5-hydroxyindoleacetic acid) were assayed to obtain an estimate of NAT HIOMT and monoamine oxidase (MAO) enzyme activities respectively Determination of [14]C 5-HIAA was considered of importance to ascertain the specific adrenoceptor effect ofthe agonist and exclude a mere inhibition of serotonin deamination. When this metabolic pathway which alternates with N acetylation of serotonin, is inhibited a "mass-action" shunt over to N-acetylation has been suggested (Axelrod, Shein and Wurtman 1969 Shein 1971). A newly synthesized β-adrenoceptor agonist KWD 2033 (Carlsson *et al* 1975) was chosen for the stimulation of pineal adrenoceptor mediated N-acetylation of [14]C-serotonin. KWD 2033 has recently been shown to have maximal efficacy and potency for stimulation of rat pineal gland β-adrenoceptors in organ culture (Bäckström 1976). Metabolite production was measured in the culture medium calculated per pineal and no compensation was made for the differences in amount of tissue between *in situ* pineal glands and transplants.

References

AXELROD, J., H. M. SHEIN and R. J. WURTMAN, Stimulation of C[14]-melatonin synthesis from C[14]-tryptophan by noradrenaline in rat pineal in organ culture. *Proc. nat. Acad. Sci.* (Wash.) 1969, 62, 544–549.

AXELROD, J., R. J. WURTMAN and S. H. SNYDER, Control of hydroxyindole O-methyltransferase activity in the rat pineal gland by environmental lighting. *J. biol. Chem.* 1965, 240, 949–954.

BÄCKSTRÖM, M., Effects of selective β adrenoceptor agonists on rat pineal gland serotonin metabolism. 1976. To be published.

B[illegible] [illegible], M., L. OLSON and Å. SEIGER, N-acetyltransferase and hydroxyindole-O-methyltransferase activity in intraocular pineal transplants. Diurnal rhythm as evidence for functional sympathetic adrenergic innervation. *Acta physiol. scand.* 1976, 96, 64–71.

BÄCKSTRÖM, M. and L. WETTERBERG, Increased N-acetylserotonin and melatonin formation induced by d-amphetamine in rat pineal gland organ culture [illegible] β-adrenergic receptor mechanism. *Acta physiol. scand.* 197[illegible], 87, 113–120.

C[illegible], W. B. and A. ROSENBLUETH, *The supersensitivity of denervated structures.* Macmillan, New York, 1949.

C[illegible], E., H. PERSSON, G. SAMUELSSON, H. SCHRÖTER and K. WETTERLIN, *Deutsche Offenlegungsschrift* [illegible] 790 731.

D[illegible] [illegible], T. and J. AXELROD, Induction and superinduction of serotonin N-acetyltransferase by adrenergic drugs and denervation in rat pineal organ. *Proc. nat. Acad. Sci.* (Wash.) 197[illegible], 69, 2208–2211.

D[illegible] [illegible], T. and J. AXELROD, Superinduction of serotonin N-acetyltransferase and supersensitivity of adenyl cyclase to catecholamines in denervated pineal gland. *Mol. Pharmacol.* 1973, 9, 612–618.

[illegible], N., J. L. WELLER and D. C. KLEIN, Development of a circadian rhythm in the activity of pineal serotonin N-acetyltransferase. *J. Neurochem.* 197[illegible], 19, 1335–1341.

G[illegible], C. and N. H. NEFF, Evidence for a specific monoamine oxidase associated with sympathetic nerves. [illegible] 1971, 10, 557–564.

H[illegible], R. and C. OWMAN, Effect of denervation and enzyme inhibition on DOPA decarboxylase and monoamine oxidase activities of rat pineal gland. *J. Neurochem.* 1965, 12, 417–429.

H[illegible], R. and Ch. OWMAN, Pineal dopa decarboxylase and monoamine oxidase activities related to [illegible] monoamine stores. *J. Neurochem.* 1966, 13, 597–605.

J[illegible], J. P., Some observations upon a new inhibitor of monoamine oxidase in brain tissue. *Biochem. Pharmacol.* 1968, 17, 1285–1297.

KLEIN, D. C., G. R. BERG and J. WELLER, Melatonin synthesis: Adenosine 3',5'-monophosphate and norepinephrine stimulate N-acetyltransferase. *Science* 1970, 168, 979–980.

KLEIN, D. C. and S. V. LINES, Pineal hydroxyindole-O-methyltransferase activity in the growing rat. *Endocrinology* 1969, 84, 1523–1525.

KLEIN, D. C. and A. NOTIDES, Thin-layer chromatographic separation of pineal gland derivatives of serotonin-[14]C. *Anal. Biochem.* 1969, 31, 480–483.

K[illegible], D. C. and J. W[illegible], Input and output signals in a model neural system: The regulation of melatonin production in the pineal gland. *In Vitro* 1970, 6, 197–204.

M[illegible], A. B. M., Electron microscopy of developing sympathetic fibres in the rat pineal body. The formation of granular vesicles. I. *Progr. in Brain Res.* (Ed. O. Eränkö) 1971, 34, 171–185.

M[illegible], R. Y., Pineal transplants to the anterior chamber of the eye. Evidence for functional reinnervation. *[illegible]* 1975, 49, 617–6[illegible].

N[illegible], C. A., D. P. CARDINALI and J. M. ROSNER, Retinal and pineal hydroxyindole-O-methyltransferases in the rat. Changes following cervical sympathectomy, pinealectomy or blinding. *Endocrinol.* 1973, 15[illegible], 1[illegible].

N[illegible], N. H., H. Y. T. YANG, C. GORIDIS and D. BIALEK, The metabolism of indolalkylamines by type A and B monoamine oxidase of brain. I. *Advances in Biochem. Psychopharmacol.* Raven Press, New York, 1974, 11, 51–58.

OLSON, L. and T. MALMFORS, Growth characteristics of adrenergic nerves in the adult rat. Fluorescence histochemical and [3]H-noradrenaline uptake studies using tissue transplantations to the anterior chamber of the eye. *Acta physiol. scand.* 1970, Suppl. 348, 1–112.

RAISZ, L., Bone resorption in tissue culture. Factors influencing the response to parathyroid hormone. *J. clin. Invest.* 1965, 44, 103–116.

SHEIN, H. M., Control of melatonin synthesis by noradrenaline in rat pineal organ cultures. In: Wolstenholme [illegible]

an extinction of HIOMT diurnal variation. Thus, eye chamber transplants behaved similarly to *in situ* pineal glands in that they are also dependent on an intact sympathetic innervation for their diurnal HIOMT variation (Axelrod Wurtman and Snyder 1965). From the present experiments it can be seen that 24 h incubation with a directly acting β-adrenoceptor agonist could increase the production of ¹⁴C MEL in denervated transplants but not in innervated transplants, although the latter displayed a dark induced rise in HIOMT activity *in oculo* The reason for this discrepancy is not readily explained Nagle, Cardinali and Rosner (1973) found that intraperitoneal injections of noradrenaline caused significant increase in pineal HIOMT activity only in ganglionectomized rats and did not affect enzyme activity in intact animals They interpreted these findings as a phenomenon of denervation supersensitivity The present observations on ¹⁴C MEL formation in denervated transplants might be similarly explained. Pineals from adult donors grafted to sympathetically innervated eyes loose some of their capacity to form ¹⁴C MEL in spite of restoration of the sympathetic innervation. It is possible that the capacity of pineal grafts from adult donors to survive as functional eye chamber transplants is less than for fetal tissues.

Innervated and denervated transplants produced ¹⁴C 5-HIAA in equally large amounts as the *in situ* pineal glands. Total MAO activity (measured in homogenates) has been reported to fall 30–50% following denervation of rat pineal glands (Snyder Fischer and Axelrod 1965 Håkansson and Owman 1966, Snyder 1968) mainly through elimination of MAO-A isoenzyme activity (Johnston 1968) which is predominantly contained in the sympathetic nerve terminals in the rat pineal (Goridis and Neff 1971 Neff *et al* 1974) Serotonin deamination was reported to decrease 70 % as compared to normal pineals when measured in homogenates of chronically denervated pineal glands (Goridis and Neff 1971) Any denervation induced decrease in ¹⁴C 5-HIAA formation was not revealed in the present experiment. This might be explained by a change of enzyme affinity for serotonin within the fraction of MAO enzyme remaining despite denervation It can be noted that Håkansson and Owman (1965) found no decrease in pineal gland MAO activity for 21 days post denervation when ¹⁴C-serotonin was used as substrate for incubations of intact glands. Bäckström and Wetterberg (1973) showed that chronically denervated *in situ* pineals cultured for 24 h in medium containing 0 25 mM ¹⁴C-serotonin, produced as much ¹⁴C 5-HIAA as did normal *in situ* pineal glands in culture.

We conclude that pineal glands transplanted into sympathetically innervated host eyes become innervated regardless of the state of innervation or lack of such at the time of operation. The present experiments lend support to the suggestion that denervated eye chamber pineal transplants might become supersensitive to aminergic stimulation and that this supersensitivity could explain the discreet NAT enzyme alternation between dark and light that was seen in denervated transplants. It is not necessary for a normally sympathetically innervated tissue ever to have been innervated to develop a "denervation supersensitivity to sympathomimetic amines.

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Local Reflex in Microcirculation in Human Skeletal Muscle

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Abstract

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The effect of venous stasis of 40 mmHg upon blood flow in human skeletal muscle was studied in four normal subjects and in two chronically sympathectomized patients. Blood flow in skeletal muscle was measured by the local ^{133}Xenon washout technique. Blood flow decreased about 30 per cent during venous stasis of 40 mmHg. In a "passive vascular bed" induced by means of histamine, blood flow decreased only by 16 per cent, indicating that the decrease in blood flow is due to a vasoconstrictor response to increase in vascular transmural pressure. The vasoconstrictor response was unaffected by spinal sympathetic blockade, but was blocked in areas infiltrated with lidocaine or with phentolamine. The vasoconstrictor response was present in the nonoperated limbs used as a control, but abolished in the denervated arms in the two chronically sympathectomized patients. The findings strongly suggest that the vasoconstrictor response in skeletal muscle is due to a local nervous mechanism involving adrenergic fibres. Thus a local reflex mechanism, most likely a sympathetic axon reflex, seems to be present in human skeletal muscle as in cutaneous and subcutaneous tissue. This indicates that about 45 per cent of the change in total vascular conductance, when a person changes from supine to upright position, is due to this local reflex mechanism operating independently of the central nervous system.

Blood flow in human skeletal muscle decreased about 50 per cent when the leg was placed in a dependent position (Baldin *et al.* 1971, Amery *et al.* 1973). This vasoconstrictor response to increase in vascular transmural pressure has also been demonstrated in human subcutaneous adipose tissue (Henriksen, Levin Nielsen and Paaske 1973) and human cutaneous tissue (Henriksen *et al.* 1973). The vasoconstrictor response has been shown to be due to a local sympathetic reflex mechanism in these tissues (Henriksen and Alsoer 1975, Henriksen 1976 a, b, c, and d, Henriksen and Sejrsen 1976 a).

The purpose of the present study was to investigate whether there is a local reflex mechanism in the microcirculation in human skeletal muscle also. If so, the local reflex mechanism might account for a great deal of the total change in vascular conductance during changes from supine to upright position.

holme, G. E. W nd J Knight (eds) *The P neal gland* Ciba Foundat Sympos. Churchill Livingstone Edinburgh and London. 1971 197 207

SNYDER, S. H J FISCHER and J AXELROD Evidence for the presence of monoamine oxidase in sympathetic nerve endings. *Biochem Pharmacol* 1965 *14* 363–365

SNYDER, S. H Development of enzyme activities and a circadian rhythm in pineal gland serotonin. E idence fo a nonretinal pathway of light to the pineal gland of newborn rats. *Ad anc Pharmacol* 1968 Part A. *6* 301 305

STRADA, S. J and B WEISS, Increased response to catecholamines of the cyclic AMP system of rat pineal gland induced by decreased sympathetic activity *Arch Biochem* 1974 *160* 197–204

TRENDELENBURG, U I Mecha isms of supersensitivity and subsensitivity to sympathomimetic amines *Pharmacol Rev* 1966. *18* 629–640.

TROWELL, O A. The culture of mature organs in a synthetic medium. *Exp cell Res.* 1959 *16* 118–147

TSUKAHARA, S. D JACOBOWITZ and A. M LATIES. Cholinergic rei nervation of iris transplants to the anterior chamber of the eye. *Amer J Ophthalmol.* 1972. *73* 394–399

WEISS, B Ontogenetic development of denyl cyclase and phosphodiesterase in rat brain. *J Neurochem.* 1971 *18* 469–477

WEISS, B and S. J STRADA, Neuroendocrine control of the cyclic AMP system of brain and pineal gland *Advanc. Cyclic Nucleotide Res* 1972. *1* 357 374.

WEISSBACH H B G REDFIELD and J AXELROD, Biosynthesis of melatonin Enzymatic conversion of serotonin to N-acetylseroto in. *Biochim biophys. Acta* (Amst.) 1960. *43* 352 353

WURTMAN, R. J F LARIN J AXELROD H M SHEIN and K ROSASCO, Formation of melatonin and 5-hydroxyindole acetic acid from 4C tryptophan by rat pineal glands in orga culture. *Nature* (Lond) 1968 *217* 953–954

ZWEIG M H and S H SNYDER, The development of serotoni and serotonin-related enzymes in the pineal gland of the rat. *Communic Behaviour Biol.* 1968 Part A. *1* 103–108

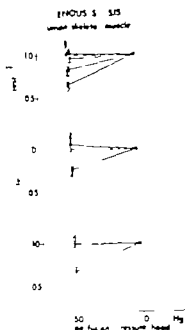


Fig 1

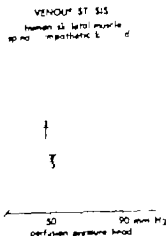


Fig. 2

Fig Relat blood flo human skeletal muscle during venous stasis of 40 mmHg, obtained 3 normal subjects plotted against perfusion pressure head (mmHg). f_{stasis} denotes perfusion coefficient obtained during venous stasis, and f_{rest} the average perfusion coefficient calculated from the values obtained just before and after the tes control, ● reas inf ltrated ith phentolamine, areas infiltrated ith lidocaine, areas infiltrated ith histamine. Vertical lines ith bars denote 1 S.E. Figures denote number of expts

Fig Relative blood flow in human skeletal muscle during enous stasis of 40 mmHg, obtained before and after spinal ympathet blockade induced by epidural anesthesia, plotted against perfusion pressure head before blockade, ● after blockade. Vertical lines ith bars denote 1 S.E. Figures denote number of expts

The cal test became negative after the epidural anesthesia had been induced and was still negativ after conclusion of the measurements, indicating that the sympathetic f bres had been blocked. Arterial blood pressure remained almost constant during the measuring period. f_{rest} obtained just after the blockade was effective was approximately 50 per cent higher than that obtained just before the blockade. Blood flow decreased 40 before (p 0.01) and 35 (p 0.05) after the blockade during venous stasis of 40 mmHg (Fig.). There was no difference in the response (p 0.7).

Lidocaine and phentolamine blocked the vasoconstrictor response to enous stasis as blood flow remained constant corresponding to a decrease in vascular resistance (Fig. 1). When lidocaine (3 10 mol l) was applied, relative blood flow ±S.E. was 0.97 _0.05 4, p 0.6

When the tissue was infiltrated with histamine, f_{rest} increased from 0.017 min to 0.56 min During enous stasis, blood flow in these areas decreased 16 per cent (p 0.001) (F g 1). This response differed significantly from that obtained in controls (p 0.001).

Methods

The experiments were carried out on four healthy subjects and two chronically sympathectomized patient operated because of manual hyperhidrosis.

Room temperature was about ..°C and remained const nt during the investigations Blood flow in th anterior tibial muscle was estimated by the local [133]Xenon washout tech ique (Larsen, Lindbjerg and Munc 1964). The γ-emissio was detected by means of NaI(Tl) sci t llation detect rs, coll mated t record on from the distal half of the labelled area in der to avoid i terference from acti ty emitted by [133]Xenon accumulating i fat tissue alo g the veins. The measurements were started 20 mi p I In order to minimu the interference f the i jection trauma I crease in vascul r transmural pressure was induced by venou stasis. A cuff was placed on the thigh o the upper arm, and the [133]Xenon washout rate constant, k measured consecutively 1) w th the cuff deflated k_{ref} 2) with the cuff i flated t 40 mmHg, k_{st} and 3) with the cuff deflated k_{ref}. Each period f measurement lasted about 3 min, nd the count rat was pri ted o t every 10 r 20 s.

I N rmals

( ) Centr l nervou blockade In order to test the possible cont b tion by central reflex mecha isms the effect of p nal sympathetic blockade pon the response to enous stasis was in estigated n one subject A catheter was placed in th epid ral space before the measurements were started 3 expt ere carried ut bef re and 3 after the ep dural anesthesia had been duced by marcaine (Henriksen nd Alsner 1975) The sweat test (Dhunér et al 1960) was performed before d after the infusion of marca ne and after the measurements were term nated

(b) Local nervous blockade The effect f local nervous blockade or lpha receptor blockade upon the response to venou stasis was studied by i jecting 1 ml of phent lam ne (10 mg/ml) 1 ml of lidocaine witho t asoconstringent (20 mg/ml 3 10^{-4} mol/l) m xed w th 0.. ml [133]Xen n n isotonic sal ne nt the anterior t bial muscle. The experiments were started 20 m later w th the subject placed upine pos tion

( ) Blockade) the asc lar smooth m scle ell A passive vascular bed was induced by inject ng 0 1 ml of histami e as the base (1 mg/ml) m xed with [133]Xenon into the terio t bial muscle The activity was recorded every 5 s, and each period of measurement lasted only 1 5 min

(d) Measurement f venous pressure Veno s pressure was measured d rectly o the dorsum of the hand duri g enou stasis in one subject placed i a supine pos tion

II Chronic sympathetic dener tion

1) The experiments were performed o the brachioradial muscle n both arms of a un laterally sympathectomized patient perated bout 9 years before The ha d the side f peration as completely dry wh le there was t ll ma ual hyperhidrosis on th other sid (co trol de) The effect of venous stas pon muscle blood flow was studied i both arms.

2) I a bilaterally sympathectomized patient, studied two years post perat ely measurement ere perf rmed on the brachiorad l muscle, and the anterio t bial muscle was used as ontrol

Calculation and stat st

Mea perfusi coefficient, f can be calculated from the Kety-equatio f k λ 100 (ml 100 g m) (Kety 1951), where k denotes the washout rat consta t m and λ the muscular tissue t blood part tion coefficient i ml g (0 7 ml/g) (Lasse et al 1964).

Relati blood flow $f_{(stasis)}/f_{(ref)}$ equals relative [133]Xeno w shout rat const nt k_{stasis} k_{ref} the tissue to blood partitio coefficient λ, assumed t be equal i the two situations $k_{(ref)}$ and f_{ref} here denot average value of the washout rate co stants (m) nd the perfusion coefficient (ml 100 g m) btained just before and after the test respectively

k_{stasis} nd $k_{(ref)}$ were compared by mea of the Stude t t test for paired samples The calculated relativ blood flow values btained d ring stasis and under the different cond tions were compared b means of the randomizatio test for unpaired samples. Lev l of sign ficance was chosen as 0 05

Results

Blood flow decreased 30 per cent during venous stasis of 40 mmHg in all 4 subjects (Figs 1 and 2)

During venous stasis the relative distribution volume of ¹³³Xenon will increase due to accumulation of blood in the distended veins, resulting in a decrease in the washout rate constant. Direct measurements of blood pressure in the superficial veins revealed that venous pressure increased close to the cuff pressure within 1 min, indicating that the major part of the increase in venous volume has occurred within this time. ¹³³Xenon solubility in blood and muscle tissue has been found to be 2.5 and 1.6 respectively relative to water (Conn 1961). Assuming that the vascular volume after 1 min of venous stasis of 40 mmHg has increased maximally from 5 to 9% of the tissue volume, the increase in relative volume of distribution would be about 3.6%. Thus the washout rate constants obtained after 2 min of stasis are then underestimated by less than 4%.

Increase in filtration of fluid out of the vascular system during venous stasis might also interfere. Assuming that the capillary filtration capacity in skeletal muscle is 0.01 ml/100 g min mmHg (Cobbold *et al.* 1963) and that the increase in mean capillary pressure is 20 mmHg during venous stasis of 40 mmHg, the error introduced by 1 min of venous stasis could be less than 1%. Even when the tissue is infiltrated with histamine, the error is insignificant, because capillary filtration capacity probably increases maximally four times (Cobbold *et al.* 1963), indicating that the error would be less than 4%.

Vasoconstrictor response

Blood flow in skeletal muscle decreased 30% during venous stasis of 40 mmHg. In a "passive vascular bed" induced by histamine, blood flow decreased by only 16% during venous stasis of 40 mmHg. Reference blood flow was approximately 39 and 2 ml/100 g min with and without histamine, respectively. With histamine the calculated vascular resistance is similar to that obtained after work to exhaustion during 10 min of ischemia, indicating that the vascular tonus was almost abolished. This difference in response between the normal and the "passive" vascular bed indicates that the decrease in blood flow during venous stasis of 40 mmHg is due to arteriolar constriction in the normal area. This is compatible with the observed increase in vascular resistance in skeletal muscle to increase in transmural pressure induced by postural changes (Balkdin *et al.* 1971, Amery *et al.* 1973).

The response was not affected by central nervous blockade, but was blocked by lidocaine and phentolamine. In these cases, blood flow remained constant during venous stasis of 40 mmHg, corresponding to a decrease in vascular resistance of 45%. The response was present in the control limbs but abolished in the denervated limbs in the chronically sympathectomized patients.

The effect of sympathectomy upon the vasoconstrictor response might be due to secondary affection of vascular smooth muscle reactivity following degeneration of sympathetic fibres. However, initiation and propagation of rhythmic activity in smooth muscles in small subcutaneous arteries in dogs were not affected (Johansson and Bohr 1966). Also, reactivity of cat portal vein to acetylcholine was not diminished by chronic sympathectomy (Johansson *et al.* 1969). In humans, autoregulation of blood flow in subcutaneous tissue was present years after sympathectomy (Henriksen 1976 b).

The blocking effect of lidocaine and phentolamine might be due to direct action on the

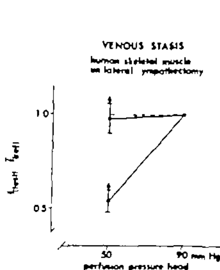


Fig. 3

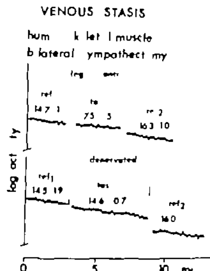


Fig. 4

Fig. 3 Relative blood flow i human skeletal muscle duri g venous stasis of 40 mmHg, obtained from the non-operated side (control) and the operated side (denervated) in a unilaterally sympathectomized patient about nine years after the operation. ○ = control side, ● = denervated side. Vertical lines with bar denote 1 S.E. Figures denote number of expts.

Fig. 4 [133]Xenon washout curves obtained before, during nd after venous stasis of 40 mmHg in a bilaterally sympathectomized patient studied bout 2 years postoperatively Upper curves obtained from the non operated leg used as control, lower curves are obtained from a denervated forearm. Figures above the curves denote the washout rate constants (k) 10^3 (min).

II *Chronic sympathetic denervation*

Blood flow decreased 45% ($p < 0.01$) in response to venous stasis on the control side but remained constant ($p > 0.8$) on the denervated side in the unilaterally sympathectomized patient (Fig. 3) The difference in response was significant ($p < 0.02$) In the other patient relative blood flow during venous stasis, $f_{test}/f_{rest} \pm$ S.E. was 0.88 ± 0.05 on the denervated forearm ($p > 0.05$) and 0.54 ± 0.04 ($p < 0.01$) on the leg used as a control. An example of the [133]Xenon washout curves obtained in this patient is shown in Fig 4.

Discussion

Washout rate

The Xenon washout rate found 20 min after the injection is influenced by accumulation of the tracer in fat cells lining the vessels (Tonnesen and Sejrsen 1967) and "veno-arterial" shunting of [133]Xenon by diffusion of intert gas in skeletal muscle (Sejrsen and Tonnesen 1972) Influence of activity emitted from [133]Xenon accumulated in these fat cells was minimized by collimating the detectors to record only from the distal part of the labelled area. Due to the "veno-arterial" shunting by diffusion, blood flow calculated from the [133]Xenon washout curve obtained 20 min after the injection at resting conditions corresponds to a value lying between 40 and 80 % of the directly measured venous outflow (Tonnesen and Sejrsen 1970)

The findings in the present study are similar to those obtained in human cutaneous tissue and human subcutaneous adipose tissue (Henriksen and Sejrsen 1976a, Henriksen and Alsner 1975, Henriksen 1976 a, b, c, d). This means that blood flow in cutaneous tissue, subcutaneous adipose tissue, and skeletal muscle decreases bout 50 % due to this local reflex mechanism, when venous transmural pressure is elevated about 25 mmHg or more.

Pleth smography

Blood flow measured by venous plethysmography might be influenced by the reflex. With a cuff pressure of 40 mmHg it took between 40 and 60 s for the venous pressure to increase t the threshold level of the reflex, 25 mmHg, indicating that the reflex is not elicited during enous occlusion plethysmography for measurements of blood flow at low venous pressures.

When the method is used for measurement of capillary filtration capacity (Mellander Öberg and Odelram 1964), possible involvement of the reflex should be considered.

Haemo-dynamic implications of the refle

1) *Postural changes* from supine to upright posit on caused an average decrease in cardiac output of 1.7 l/min (Hanson, T baken and Levy 1968), corresponding to a decrease in total ascular conductance of about 22 %. Assuming that enous transmural pressure increases more than 25 mmHg in tissues corresponding to 50 of the b.wt. and that the average decrease in blood flow is 2 ml/100 g min in these tissues, the local reflex mechanism ould account for a decrease in ascular conductance of bout 10 %, which is 45 of the total change. This means that about 45 of the postural changes in vascular tonus is due t this local reflex, occurring independently of the central nervous system. The remaining 55 are probably due to central reflex mechanisms elicited from baroreceptors located in the large arteries, central eins, and heart (Roddie and Shepherd 1957, Beiser *et al.* 1970, Rowell, Wyss and Brengelmann 1973).

) *Autoregulation of blood flow* (i.e. maintenance of constant blood flow during changes in arterial perfusion pressure head) became evident when the reflex was blocked, indicating that autoregulation is due to intrinsic mechanisms.

References

AMERY A., H. BOSSAERT, M. DERUYTTERE, L. VANDERLINDEN and M. VERSTRAETE, Influence of body posture on leg blood flow. *Scand. J. clin. lab. Invest.* 1973, *31* Suppl 128, 29

BALLERS L. I. C. E. G. L. NOGAARD, J. LUNDWALL and S. MELLANDER, Changes in disappearance of [133]Xenon from the anterior tibial muscle in man induced by immersion in water and by shift in body position. *Aerospace Med.* 1971, *4* 489–493

BEISER G. D., R. ZELIS, S. E. EPSTEIN, D. T. MASON and E. BRAUNWALD, The Role of Skin and Muscle Resistance Vessels in Reflexes Mediated by the Baroreceptor System. *J. clin. Invest.* 1970, *49* 225–231

COBBOLD A., B. FOLKOW, I. KJELLMER and S. MELLANDER, Nervous and local chemical control of pre-capillary sphincters in skeletal muscle as measured by changes in filtration coefficient. *Acta physiol. scand.* 196 , *57* 180–19

CONN, H. L. Jr., Equilibrium distribution of radioxenon in tissue: Xenon-hemoglobin association curve. *J. appl. Physiol.* 1961, *1* 1065–1070

DHUNER, K. G., S. EDWARDS and A. WILHELM, Ninhydrin Test — an objective method for testing local anaesthetic drugs. *Acta anaesth. scand.* 1960, *4* 189–198.

vascular smooth muscle cells affecting myogenic reactivity Lidocaine (3 10^{-4} m
did not affect myogenic activity in the rat portal vein, but blocked inpulse transmissio
sympathetic fibres effectively (Johansson and Ljung 1967) In this concentration, lidoc
blocked the vasoconstrictor response effectively In two experiments phentolamine, 5 1
mol/l, was applied. Even in this low concentration phentolamine blocked the vasoconstri
response to venous stasis induced 15 min after the injection Four control expts with
jection of isotonic saline performed in order to test the effect of the injection trauma indica
that this trauma was without effect 15 min after the injection Phentolamine (5 10 mo
did not affect myogenic activity in the rat portal vein (Johansson and Mellander 1975).

The effect of lidocaine and phentolamine is, therefore, most likely due to a blockade
sympathetic adrenergic vasoconstrictor fibres indicating that the vasoconstrictor respo
is due to a local nervous mechanism

Folkow and Öberg (1961) observed that venous stasis of 10 mmHg induced a decre
in blood flow in skeletal muscle in reserpinized cats, indicating that vascular reactivity
still present in more or less complete catecholamine depleted tissue. The duration of t
periods of measurement was less than 40 s. Jones and Berne (1964) observed a transie
decrease in blood flow in the thigh muscle of dog during venous stasis of 20 mmHg, lasti
about 40 s whereafter blood flow almost returned to the level before the period of stas
Lundvall and Mellander (1976) made similar observations in skeletal muscle of cat durin
external negative pressure of 40 mmHg. These observations suggest an intrinsic vascul
response to increase in vascular transmural pressure

In humans a steady decrease in blood flow was observed during the venous stasis
days after sympathectomy this response was abolished indicating that the vasoconstrict
response to venous stasis in humans rather is neurogenic than an intrinsic vascular respon

The findings in the reserpinized cats (Folkow and Öberg 1961) is in agreement with th
observation that autoregulation in subcutaneous tissue was retained years after the symp
thectomy (Henriksen 1976 b) This observation does not exclude an overlying nervo
reinforcing mechanism.

When increase in venous transmural pressure during lowering of the limb by 40 cm
was prevented by letting the subject tip his foot, the vasoconstrictor response was blocke
in subcutaneous tissue as vascular resistance increased only by 48 % corresponding to th
increase in arterial perfusion pressure head (autoregulation) (Henriksen and Sejrsen 1976 b)
The abolishment of the vasconstrictor response during exercise might be due to liberatio
of metabolites in subcutaneous tissue affecting myogenic activity However elevation o
venous transmural pressure during exercise by inducing venous stasis elicited an increase
in vascular resistance of the same magnitude as in the lowered, resting leg. In patients with
venous insufficiency exercise did not prevent the increase in venous transmural pressure
and did not abolish the vasoconstrictor response Thus the abolishment of the vasoconstrictor
response to lowering during exercise, is probably due to prevention of increase in venous
transmural pressure, indicating that the vasoconstrictor response depends upon an impulse
transmission from veins to arterioles. This furthermore strongly suggests that the vaso-
constrictor response is due to a local nervous mechanism, probably a sympathetic axon
reflex.

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Efflux of 5-Hydroxytryptamine from Synaptosomes of Rat Cerebral Cortex

By

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Received 30 April 1976

Abstract

Ross, S. B. and D. Kelder. *Efflux of 5-hydroxytryptamine from synaptosomes of rat cerebral cortex.* Acta physiol scand. 1977 99 27–36.

The efflux of ^{3}H-5-HT from a crude synaptosome preparation of the cerebral cortex of reserpinized rats was examined. The synaptosomes were loaded with ^{3}H-5-HT by pre-incubation of the homogenate in presence of pargyline and desipramine in order to inhibit deamination of 5-HT and uptake into noradrenergic neurones. The synaptosomes were collected by centrifugation, washed and resuspended in 0.25 M sucrose. No spontaneous efflux of 5-HT was detectable at 0°C but marked efflux was observed at 27°C and 37°C. 4-Chloroamphetamine, low external Na concentration, ouabain and the depolarizing agent veratridine markedly accelerated the initial (5 min) efflux. Inhibitors of the neuronal 5-HT uptake, e.g. chlorimipramine, H 102/09 and A 23189 antagonized the 5-HT efflux evoked by these means, whereas desipramine, which is a poor inhibitor of the 5-HT uptake, had only slight effect on the 5-HT efflux. It is suggested that 5-HT can be actively transported out from the synaptosomes by the reversed 5-HT uptake mechanism.

Key words: 5-Hydroxytryptamine efflux, 4-chloroamphetamine, chlorimipramine, H 102/09, A 23189

The physiological importance of the neuronal membranal uptake mechanisms for the biogenic monoamines is generally recognized and accepted. Due to the hydrophilic nature of these amines they slowly pass the neurone membranes by diffusion and active uptake mechanisms are therefore necessary for a rapid transport of these amines through the membranes. It is also a general accepted view that the indirectly acting sympathomimetic amines exert their action by releasing the biogenic monoamines from intraneuronal binding sites (Trendelenburg 1972). From pharmacological observations it has been concluded that this release is different from that produced by nerve stimulation (Furchgott *et al.* 1963). Recently Thoa *et al.* (1975) found that the release of noradrenaline by the sympathomimetic amines in contrast to that of nerve stimulation in the isolated vas deferens was not joined by release of the enzyme dopamine β-hydroxylase. If the release of the biogenic amines does not occur with an exocytosis mechanism the problem remains how the released amines pass the neurone membranes outwardly since the same barrier exists in both directions. Until recently this problem has been overlooked, although it was suggested as possible that

FOLKOW, B. and B. ÖBERG, Autoregulation and basal tone in consecutive vascular sections of the skeletal muscles in reserpine treated cats. *Acta physiol. scand.* 1961. 53. 105–113.

HANSON, J. S., B. S. TALAKIN and A. M. LEVY. Comparative exercise-cardio-respiratory performance of normal men in the third, fourth, and fifth decades of life. *Circulation* 1968. 37. 345–360.

HENRIKSEN, O. Effect of somaesthetic denervation upon the vasoconstrictor response in human subcutaneous tissue. *Acta physiol. scand.* 1976 a. 96. 431–432.

HENRIKSEN, O. Effect of chronic sympathetic denervation upon blood flow in human subcutaneous tissue. *Acta physiol. scand.* 1976 b. 97. 377–384.

HENRIKSEN, O. Local nervous mechanism in regulation of blood flow in human subcutaneous tissue. *Acta physiol. scand.* 1976 c. 97. 385–391.

HENRIKSEN, O. Local reflex in microcirculation in human subcutaneous tissue. *Acta physiol. scand.* 1976 d. 97. 447–456.

HENRIKSEN, O. and T. ALSNER. Effect of spinal sympathetic blockade upon local regulation of blood flow in subcutaneous tissue. *Acta physiol. scand.* 1975. 95. 83–88.

HENRIKSEN, O. and P. SEJRSEN, Local reflex in microcirculation in human cutaneous tissue. *Acta physiol. scand.* 1976 a. 98. 227–231.

HENRIKSEN, O. and P. SEJRSEN. Effect of "veinpump" activation upon venous pressure and blood flow in human subcutaneous tissue. *Acta physiol. scand.* 1976 b. Suppl. 440. Abstract No. 197. p. 179.

HENRIKSEN, O., S. L. NIELSEN, W. P. PAASKE and P. SEJRSEN. Autoregulation of blood flow in human cutaneous tissue. *Acta physiol. scand.* 1973. 89. 538–543.

JOHANSSON, B. and B. LJUNG, Spread of excitation in the smooth muscle of the rat portal vein. *Acta physiol. scand.* 1967. 70. 312–322.

JOHANSSON, B., B. LJUNG, T. MALMFORS and L. OLSON, Denervation supersensitivity in the sparsely innervated smooth muscle of the portal vein. *Acta physiol. scand.* 1969. Suppl. 330. 58.

JOHANSSON, B. and S. MELLANDER, Static and dynamic components in vascular myogenic response to passive changes in length as revealed by electrical and mechanical recordings from the rat portal vein. *Circulat. Res.* 1975. 36. 76–83.

JONES, R. D. and R. M. BERNE. Local regulation of blood flow in skeletal muscle. *Circulat. Res.* 1964. 15. Suppl. I. 30–38.

LASSEN, N. A., I. F. LINDBJERG and O. MUNCK, Measurement of blood flow through skeletal muscle by intramuscular injection of Xenon 133. *Lancet* 1964. I. 686.

LUNDVALL, J. and S. MELLANDER. 1976. Personal communication.

MELLANDER, S., B. ÖBERG and H. ODELRAM. Vascular adjustments to increased transmural pressure in cat and man with special reference to shifts in capillary fluid transfer. *Acta physiol. scand.* 1964. 61. 34–48.

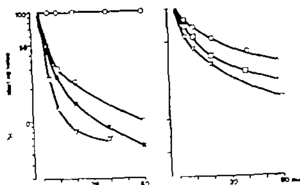
RODDIE, J. C. and J. T. SHEPHERD, The effects of carotid artery compression in man with special reference to changes in vascular resistance in the limbs. *J. Physiol.* (Lond.) 1957. 139. 377–384.

ROWELL, L. B., C. R. WYSS and G. L. BRENGELMANN, Sustained human skin and muscle vasoconstriction with reduced baroreceptor activity. *J. appl. Physiol.* 1973. 5. 639–643.

SEJRSEN, P. and K. H. TØNNESEN, Shunting by diffusion of inert gas in skeletal muscle. *Acta physiol. scand.* 1972. 86. 82–91.

TØNNESEN, K. H. and P. SEJRSEN, Inert gas diffusion method for measurement of blood flow. Comparison of bolus injection to directly measured blood flow in isolated gastrocnemius muscle. *Circulat. Res.* 1967. 20. 552–564.

TØNNESEN, K. H. and P. SEJRSEN, Washout of ^{133}Xenon after intramuscular injection and direct measurement of blood flow in skeletal muscle. *Scand. J. clin. Lab. Invest.* 1970. 25. 71–81.



1 Effl of ³H-5-HT from cerebral cortical synaptosomes of reserpinized rats. Synaptosomes were loaded h H 5-HT by incubation of cortical homogenat th H 5-HT (1 10⁻ M) in presence of M pargyline and 10 M desipramine as described in Method. The washed crude synaptosome suspension (100 µl) containing 227+3 (4) pmol H-5-HT/g original tissue as incubated in 2.0 ml Krebs Henseleit buffer or modified sucrose containing buffer low in N (26 mM). The efflux of H 5-HT determined by analyzing the radioactiv y in the synaptosomal pellets after arious imes of incubation and expressed per cent of the starting alue. Each alue is the mean of 4 determinations, all h S E of 4 or less A Incubation 0 C (broken lines) and 37 C (hole lines) B Incubation t Normal medium ( ), medium low in Na ( ) 4-Chloroamphetamine, 5 10⁻ M ( ) H 102 09 10 M ( )

Results

Effl of H 5-HT from rat brain synaptosomes at arious temperatures

The time course of the efflux of H 5-HT from the cortical synaptosome preparation was studied t three different temperatures and at various incubation conditions (Fig. 1) At 0 C almost no efflux was detectable during the time examined. At 37°C the efflux of 5-HT from the synaptosomes was rapid and addition of 4-chloroamphetamine (5 10 M) to the incubation medium caused a further increase in the efflux (Fig 1 A). At 27 C the rate of efflux as slower than at 37 C (F g 1 B) The data obtained give a Q_{10} value of 2.5 for the initial effluxes observed t these two temperatures. In an incubation medium with low Na (substituted with sucrose) the efflux was more rapid than in the normal incubation medium Addition of ouabain (5 10 M) and simultaneous omission of K in the incubation fluid also accelerated the efflux of H 5-HT (T ble II). The depolarizing agent veratridine (5 10 M) caused pronounced increase in the 5-HT efflux at 37 C (70 9 ± 1 6 pmol g 5 min 4 P 0 001) but no effect was observed at 0 C The effect by veratridine was antagonized by tetrodotoxin (1 10⁻⁶ M) (69 ± 5 inhibition, n 4 P 0 01) but had no effect on the 5-HT efflux evoked by low external Na (0% inhibition). Omission of Ca in the incubation medium, did not influence on the veratridine evoked efflux (71 3 1 1 pmol g,5 min 4 P 0 05).

The rapid spontaneous efflux of 5-HT at 37 C may suggest that the synaptosomes are not stable under these incubation conditions but are broken and release their content into the medium This possibility was examined by testing the ability of the synaptosomes to accumulate ³H 5-HT The synaptosomes were prepared exactly as for the efflux experiments

cocaine may antagonize the indirectly acting amines by inhibiting the outward passage o noradrenaline through the neurone membrane (Ross *et al* 1968 Ross and Renyi 1971) Experimental observations have shown that the efflux of noradrenaline from rat hear slices and slices of atria from reserpinized rabbits is an active energy dependent transport which is inhibited by desipramine and cocaine (Blazkowski and Bogdanski 1972, Paton 1973) Using bretylium as a model compound for the membranal noradrenaline transport mechanism in noradrenergic neurones of the rat vas deferens we have observed that the compound is transported actively outwardly as well as inwardly by a desipramine sensitive mechanism (Ross 1976 a, Ross and Kelder 1976) In the present study we have examined if a similar outward transport mechanism exists for 5-HT in central 5-HT neurones.

Materials and Methods

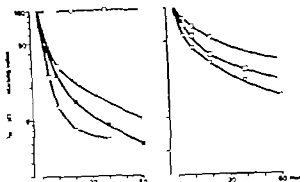
Crude synaptosomes from reserpinized male Sprague-Dawley ts (150 180 g) were prepared from the cerebral cort according to the method f Snyder and Coyle (1969) Cerebral cortical lices ere homogenized in 10 ol mes of ice-ch lled 0 .3 M sucrose a Potter El ehjem glass homogenizer The homogenate was centrif ged at 800 g for 10 m n. The supernat t (8 ml) was incubated for 15 min at 37 C w th 15 4 ml f Krebs Henseleit s buffer pH 7 4 contain ng .4 10⁻⁴ M pargyl ne 1 10 M desipramine (to inhibit uptake f 5-HT noradrenergic neurones), 1 1 10 M ascorbic acid 1 3 10⁻ M EDTA nd 5 4 10⁻³ M glucose H 5-HT was then dded givi g a f nal concentration of 1 10⁻ M and the incub tion was continued for 20 min The ncubation mi ture was centrif ged for 20 min at 16 000 g a Sorv ll RC3 centrifuge. The t bes and pellets were w shed 3 t mes with 5 ml of 0 25 M sucrose solution The pellet were re-homogenized in 8 ml of 0.25 M sucrose and centrif ged at 16 000 g for 20 min The pellet btained were homogen zed in 8 ml of 0.25 M sucrose and this synaptosome su pension used for the efflux experiments. The uptake of H 5-HT n synaptosomes wa determined as described previously (Ross and Reny 1975).

The efflu of H 5-HT from the ynaptosomes was determined under the follow g experimental conditions A aliquot of 100 μ l of the ynaptosome suspen io added t 1 9 ml of Krebs buffer or modified buffer co taining 2 4 10⁻⁴ M pargyline, 1 1 10 M ascorbic cid, 1 3 10⁻ M EDTA nd, if present the test compound in PVC centrif ge t be The incubation was perf rmed n water bath t 37 C d otherwise s ot st ted Immediat ly fter ncubatio the t bes were centrif ged at 16 000 g for 20 min The pellet and the tubes were washed w th 5 ml of cold sal ne solution nd the t bes ere rinsed from water drips w th tissue paper The pellets were dissolved 1 0 ml of Soluene-350 (Packard) at oom temperature for one ho Ten ml of the scint llation liquid were added and the radioactive measured i a liq id scint llat on pect ometer St dards of H 5-HT and bla k were run sim lt neously The amount of radioactivity the pellet s expressed n pmol H 5-HT g rginal tissue C trol experiments, in which 5-HT was separated from deam nated metabolites by th layer chromatography (Ross and Ren 1969), showed that after 0 and 60 m ncubation the mount of H 5-HT the pellet and in the medium wa 95 of the total rad oact ity Reserpine (5 mg/kg p) was injected 18 h prior t the expts.

The rmal ncubation med um (Krebs-Henseleit buffer) cont ned 117 mM NaCl, 5 4 mM KCl, 0 6 mM MgSO 2 6 mM CaCl 1 1 mM N H PO nd 25 mM N HCO nd as eq f brated with O_2 containing 6 5 of CO gn g pH of 7 4 I some experiment this buffer w mod fied by bstituting N Cl w th 0.25 M sucrose or 117 mM LiCl giv ng Na concentration of 26 mM

Compounds

5-Hydroxytryptamine- H(G) creat nine sulphat (10.7 Ci/mmol) was purchased from the Radiochem al Centre Amersham, England. Pargyline was gift from Abbott Lab Inc d chlorimipramine nd desipramine from J R Geigy A G H 10.709 ((Z)-3-(4-bromophenyl)-N,N-dimethyl-3-(3-pyridyl)allylamine d hydrochloride) and A .3189 (-aminopropanoic acid 1-(4-chlorophenyl)- -meth l-2-propylester hydrochloride) were sy thesized in the Astra laboratories.



F 1 Efflux of ³H-5-HT from cerebral cortical synaptosomes of reserpinized ra Synaptosomes er loaded ith ³H-5-HT by incubation of cortical homogenat ith ³H 5-HT (1 10⁻ M) presence of 10⁻ M pargyline and 3 10⁻ M desipramine described in Method The washed crude synaptosome suspension (100 μl) containing 227 3 (4) pmol H-5-HT g original tissue incubated in 0 ml Krebs Henseleit's buffer or modified sucrose containing buffer low in Na (26 mM) The efflux of H 5-HT is determined by analyzing the radioactivity in the synaptosomal pellets after arious mes of incubation and expressed per cent of the starting alue Each loa he mean of 4 determinations, all h S E of 4 or less A Incubation at 0 C (broken lines) and 37 C (hole lines B Incubation at (Normal medium ( ), medium low in Na ( ) 4-Chloroamphetamine, 5 10 M ( ) H 10 09 10 M ( )

Results

Efflux of H 5-HT from rat brain synaptosomes at various temperatures

The time course of the efflux of H 5-HT from the cortical synaptosome preparation was studied at three different temperatures and at various incubation conditions (Fig. 1) At 0 C almost no efflux was detectable during the time examined At 37 C the efflux of 5-HT from the synaptosomes was rapid and addition of 4-chloroamphetamine (5 10 M) to the incubation medium caused further increase in the efflu (Fig. 1 A). At 27 C the rate of efflux was slower than at 37 C (Fig 1 B). The data obtained give a Q_{10} alue of 2.5 for the total effluxes bserved at these two temperatures. In an incubation medium with low Na (substituted with sucrose) the efflux was more rapid than in the normal incubation medium Addition of ouabain (5 10⁻⁴ M) and simultaneous omission of K in the incubation fluid also accelerated the efflux of H-5-HT (Table II). The depolarizing agent eratridine (5 10⁻⁴ M) caused pronounced increase in the 5-HT efflux at 37°C (70.9 ± 1.6 pmol/g,5 min 4 P 0.001) but no effect was observed at 0 C. The effect by eratridine was antagonized by tetrodotoxin (1 10⁻⁴ M) (69 ÷ 5 inhibition, 4 P 0 01) but had no effect on the 5-HT efflux evoked by low external Na (0 inhibition) Omission of Ca in the incubation medium, did not influence on the eratridine evoked efflux (71 3 1 1 pmol/g/5 min n 4 P 0.05).

The rapid spontaneous efflux of 5-HT at 37°C may suggest that the synaptosomes are not stable under these incubation conditions but are broken and release their content int the medium This possibility was examined by testing the ability of the synaptosomes t accumulate ³H 5-HT The synaptosomes were prepared exactly as for the efflux experimen

cocaine may antagonize the indirectly acting amines by inhibiting the outward passage of noradrenaline through the neurone membrane (Ross *et al* 1968 Ross and Renyi 1971) Experimental observations have shown that the efflux of noradrenaline from rat heart slices and slices of atria from reserpinized rabbits is an active energy dependent transport which is inhibited by desipramine and cocaine (Blazkowski and Bogdanski 1972, Paton 1973) Using bretylium as a model compound for the membranal noradrenaline transport mechanism in noradrenergic neurones of the rat vas deferens we have observed that this compound is transported actively outwardly as well as inwardly by a desipramine sensitive mechanism (Ross 1976 a, Ross and Kelder 1976) *In the present study we have examined if a similar outward transport mechanism exists for 5-HT in central 5-HT neurones.*

Materials and Methods

Crude synaptosomes from reserpinized male Sprague-Dawley rats (150 180 g) were prepared f m the cerebral cortex accord ng to the method of Snyde and Coyle (1969). Cerebr l cortical slices ere homogenized i 10 ol mes of ice-ch lled 0 .3 M sucrose n a Potter Elvehjem glass homogenizer The homogen te was centrif ged at 800 g for 10 min The supernata t (8 ml) was incubated for 15 min at 37 C w th 15 4 ml of Krebs-Henseleit buffer pH 7 4 co tain g 4 10^{-4} M pargyl ne 1 10 M despr mine (t inh bit pt ke of 5-HT in oradrenergic neurones), 1 1 10 M ascorbic acid, 1 3 10^{-4} M EDTA nd 5 4 10^{-4} M glucose. H 5-HT was then added givi g a f nal c ncentration of 1 10 M and the incubation was cont ued fo 20 min The ncubation m t re was centrif ged f 20 m n t 16 000 g n a Sorv ll RC3 cent fuge. The t bes nd pellets were washed 3 t mes with 5 ml of 0 25 M sucrose solution The pellets were re homogenized n 8 ml of 0.3 M sucrose and cent fuged at 16000 g fo 20 min. The pellet obt ined were hom genized in 8 ml of 0.3 M sucrose a d this ynaptosome suspension used for the efflux experiments. The uptake of H 5-HT n synaptosomes was d term ned described previously (Ros and Renyi 1975)

The efflux of H 5-HT from the ynaptosomes was determined under the follow ng experimental conditions. A aliquot of 100 µl of the synaptosome suspension added to 1 9 ml f Krebs buffer or a modified buffer conta n ng .4 10^{-4} M pargyline 1 1 10 M scorbic acid 1 3 10 M EDTA and, f present, the test compo nd PVC cent fuge t be The incubat w perf rmed in a water bath at 37 C if otherwise is not stated Immediately after ncubation the tubes were cent f ged t 16 000 g for 20 min The pellets and the tubes were washed w th 5 ml of cold saline solution and the t bes ere rinsed from w ter drops with tissue paper The pellets were dissol ed 1 0 ml of Soluene-350 (Packard) at oom temperat re for one hou Ten ml of the sci t llatio l q d were dded nd the rad oactive measured in l quid sci tillation spectrometer St ndards of H 5-HT and blank were run sim lt neously The amount of radioact ity in the pellets s expressed in pmol H 5-HT/g orig al t sue Con rol experiments, in which 5-HT was separated from deaminated metabol tes by th n layer chromatography (Ros nd Renyi 1969), sho ed that fter 0 and 60 m incubation the mount of H 5-HT n the pellet and in the medium was 95 of the total radioacti ty Reserpine (5 mg/kg p) wa njected 18 h prior t the expt

The normal ncubation med u (Krebs-Henseleit buffer) contained 117 mM NaCl 5 4 mM KCl 0 6 mM MgSO .6 mM C Cl 1 1 mM N H PO nd 5 mM N HCO_3 and w equil br ed th O co taining 6.5 of CO_2 gi ing pH of 7 4 In some experiment this buffer as modified by but using N Cl w th 0 .3 M sucrose o 117 mM LiCl gi ing a N concentration of 26 mM

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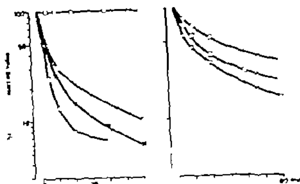


Fig. 1. Efflux of H 5-HT from cerebral cortical synaptosomes of reserpinized rat. Synaptosomes were loaded with H 5-HT by incubation of cortical homogenate with H 5-HT (1 × 10⁻⁴ M) in presence of 10⁻⁴ M pargyline and 3 × 10⁻ M desipramine as described in Methods. The washed crude synaptosomal suspension (100 ml) containing 27 3 (4) pmol H 5-HT g original tissue was incubated 0 ml Krebs Henseleit buffer or modified sucrose containing buffer low in Na (26 mM). The efflux of H 5-HT was determined by analyzing the radioactivity in the synaptosomal pellets after known times of incubation and expressed in per cent of the starting value. Each value is the mean of 4 determinations, all with S.E. of 4 or less. A Incubation at 0 C (broken lines) and 37 C (whole lines). B Incubation at 27 ... medium ... sodium low in Na ( ) 4-Chloroamphetamine 5 10 M ( ) H 10 09 10 M ( )

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The rapid spontaneous efflux of 5-HT at 37 C may suggest that the synaptosomes are not stable under these incubation conditions but are broken and release their content into the medium. This possibility was examined by testing the ability of the synaptosomes to accumulate ³H-5-HT. The synaptosomes were prepared exactly as for the efflux experiments

TABLE I Inhibition of the efflux of ^{3}H 5-HT from synaptosomes evoked by 4-chloroamphetamine (5 10^{-} M), low external Na+ (26 mM NaCl replaced by 250 mM sucrose) and by veratridine (5 10 M). The cortical synaptosomes were loaded with H 5-HT (1 10^{-} M) in presence of 2.4 10^{-} M pargyline and 3 × 10^{-} M desipramine for 20 min. The efflux of ^{3}H 5-HT was determined by incubating 100 μl of the washed suspension in .0 ml Krebs buffer or modified buffer for 5 min at 37°C. The difference in the amount of radioactivity in the pellet in absence and presence of the efflux inducer was taken as the evoked efflux. The inhibition of the evoked efflux was calculated in per cent. Each value is the mean ± S.E. of 4 determinations.

Compound	Conc. M	Inhibition 4-Chloro-amphetamine	Low Na	Veratridine
H 102/09	2.5 10^{-}	14±2	—	—
	2.5 10^{-}	48±2	61±6	59±6
A ‿3189	3.4 10^{-4}	48±4	60±14	69±5
Chlorimipramine	2.9 10^{-}	—	56±11	43±3
	2.9 10^{-}	79±3	89±5	63±3
Desipramine	3.0 10^{-}	12±3	70±5	19±4
	3.0 10^{-4}	—	9±3	40±5
M llicaine	‿9 10^{-}	—	8±4	82±3

0.05 > P > 0.01   0.01 > P > 0.001   P 0.001 (Student's t-test)

with the exception that H 5-HT was omitted in the pre-incubation of the homogenate. The uptake of ^{3}H 5-HT for a period of 5 min was examined at times corresponding to the start of the efflux experiment and after 60 min incubation at 37°C. The accumulation decreased from 64.2±9.0 (n 5) to 16.8±1.8 (n = 5) pmol/g tissue/min incubation, i.e. the uptake after 60 min incubation was only 26% of that at zero time. Thus, the partially purified synaptosomes had lost a large part of their capacity to accumulate H 5-HT by the incubation.

Effect of uptake inhibitors on the efflux of H 5-HT

The effect of H 102/09 a selective inhibitor of the membranal 5-HT uptake (Ross et al. 1976) on the efflux of H 5-HT from the synaptosomes at 37°C was examined (Fig. 1 A). H 102/09 itself caused an increase of the spontaneous efflux, which was more marked after prolonged incubation. However it significantly inhibited the initial increase of the efflux produced by 4-chloroamphetamine, by low external Na and by veratridine (Table I). Chlorimipramine (Shaskan and Snyder 1970) and A 23189 (unpublished observations) are two other potent inhibitors of the 5-HT uptake. As shown in Table I these compounds inhibited the efflux of H 5-HT induced by the various means examined. Desipramine which is a potent inhibitor of the NA uptake but a rather poor inhibitor of the 5-HT uptake (Ross and Renyi 1975) had much less effect in inhibiting the 5-HT efflux. At the higher concentration of desipramine examined the effect on the veratridine induced efflux was larger than on that evoked by low external Na. The same discrimination between the efflux evoked by veratridine and low external Na was observed for the local anesthetic agent millicaine (Table I).

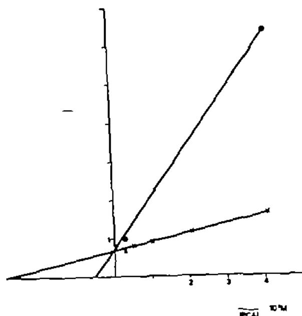


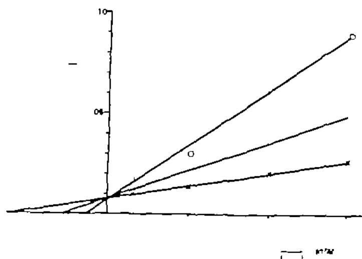
Fig. 2. Double reciprocal plot of the increase of the ³H-5-HT efflux caused by 4-chloroamphetamine (PCA). Synaptosomes were prepared and loaded with ³H-5-HT as described in the legend of Fig. 1. The incubation time was 5 min. The velocity of efflux (v) was determined as the difference of the efflux at 37°C and 0 C and was expressed in pmol/g/min. The concentration of H 102/09 (●) was 2.5 × 10^{-7} M. Each value is the mean of 4 determinations.

Kinetics of the antagonism of H 102/09 on the 4-chloroamphetamine induced increase of ³H 5-HT efflux

The kinetics of the antagonism of H 102/09 on the increased ³H 5-HT efflux produced by 4-chloroamphetamine was examined according to the method of Lineweaver and Burk (1934). The increased initial (5 min) efflux caused by 5 different concentrations of 4-chloroamphetamine (2.4 × 10^{-6} M–3.9 × 10^{-} M) were determined in absence or presence of H 102/09 (2.5 × 10 M). The result of an experiment is shown in Fig. 2. H 102/09 inhibited competetively the effect of 4-chloroamphetamine. The apparent K_m for 4-chloroamphetamine in increasing the efflux was 4.6 × 10^{-6} (mean of 2 determinations. 5.4 × 10^{-6} M and 3.7 × 10^{-6} M). The inhibitor constant (K_i) for H 102/09 was 1.1 × 10^{-7} M (mean of 1.5 × 10^{-7} M and 6.1 × 10^{-8} M). Both these values are close to the corresponding K_i values for the inhibition of the 5-HT uptake in the same synaptosome preparation being 1.2 × 10 M for 4-chloroamphetamine and 1.8 × 10^{-7} M for H 102/09 (Fig. 3). The apparent K_m value for 5-HT was in these experiments 8 × 10^{-8} M.

Effect of ouabain on the increase in efflux produced by 4-chloroamphetamine

It was of interest to examine, if ouabain antagonized the increase in efflux produced by 4-chloroamphetamine, since if so, it should indicate that 4-chloroamphetamine is actively transported by a Na⁺ dependent mechanism into the synaptosomes before it releases 5-HT



F g 3 Double rec procal plot f th uptake of H 5-HT c rebral cortical synaptosomes from reserpinized rats. The incubation t me was 5 mi The uptak elocity ( ) was determined as the d fference t 37 C nd 0 C a d was expressed pmol/g/min. The co centratio of H 10,/09 ( ) was 2 5 10 M nd that of 4-chl roamphet m e (O) 5 10 M Each value is the mean of 4 determi t ns

intraneuronally The synaptosomes were simultaneously incubated with ouabain in a medium free of K and 4-chloroamphetamine at low concentration (1 10 M) in order to be within an adequate concentration range for the potential uptake (Table II). The effect of this combination was larger than that of 4-chloroamphetamine and ouabain alone, which indicates an additive effect

Kinetic determination of the Na dependence of the 5 HT transport

Since both influx and efflux of 5-HT in synaptosomes are influenced by Na (Bogdanski *et al* 1968) it was of interest to examine kinetically the nature of the Na dependence This was best performed by working with the uptake system, since the 5-HT concentrations can then be properly controlled In this experiment we used non-reserpinized rats and the crude cortical homogenate obtained after low speed centrifugation (800 g) The determination of the uptake of H 5-HT (3 10 M 2 10 M) by the synaptosomes in the homogenate was performed in normal buffer and in buffer low in Na (26 mM) by replacing NaCl with 117 mM LiCl in order to keep the Cl concentration unchanged The double reciprocal plots (Fig. 4) show that the affinity of 5-HT for the uptake sites was decreased by the low

TABLE II The combined effect of uaba (5 10 M) no ext rnal K and 4-chloroamphetamine (1 10 M) on the efflux of H 5-HT f m cerebral rtical ynaptosomes of the ra The incubation w perf rmed f 5 min. Each lue is mea S E f 8 determination

	ΔEfflu pmol/g min + S E.	p
1 Ouabain + 0 K	5 0 _ 0 3	
2 4-Chloroamphetamine	10 2 + 0.3	
3 4-Chloroamphetamine + ouaba 0 K	12.8 + 0 1	0 001 (1)

Student s t-test

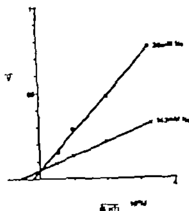


Fig 4 Double reciprocal plot of the uptake of 5-HT in homogenate of rat cerebral cortex (non-reserpinized) at two different Na concentrations The incubation time was 1 min The uptake velocity ( ) as determined as described in the legend of Fig 3

external Na concentration, whereas V_{max} was unchanged. The apparent K_m value for normal Na concentration (1 7 10⁻ M) was in this experiment twice higher than that obtained with the more purified synaptosomes obtained from reserpinized rats.

Discussion

The marked spontaneous efflux of ³H-5-HT from the cerebral cortical synaptosomes observed in this study is in accordance with the findings by Bogdanski *et al* (1968). Since it was temperature dependent it is probably due to metabolic processes. Bogdanski *et al.* (1968) found that the labelled 5-HT decreased more rapidly than the endogenous 5-HT in the synaptosomes and proposed that dilution of the labelled amine with newly synthesized 5-HT was one reason for the decline in the labelled 5-HT No attempt to test this hypothesis with an inhibitor of the 5-HT synthesis has been performed. Another possible mechanism is that the synaptosomes are instable under the incubation conditions employed, are broken and release their contents out to the medium. The loss of a large part of the capacity of the synaptosomes to accumulate 5-HT after 60 min incubation at 37°C indicates that a part of the synaptosomes deteriorate in some way This may at least partially contribute to the rapid spontaneous efflux of 5-HT observed at this temperature.

In spite of this spontaneous efflux of 5-HT very constant and reliable values were obtained hen determining the initial 5 min efflux evoked by 4-chloroamphetamine, low external Na ouabain and veratridine. Since this evoked 5-HT efflux was temperature dependent and as antagonized by uptake inhibitors it is probably an active outward transport of 5-HT The observation that H 102/09 which is a selective inhibitor of the neuronal 5-HT uptake (Ross *et al.* 1976), accelerated the 5-HT efflux but antagonized the efflux evoked by arious means appears somewhat conflicting The acceleration of the efflux is understood, if it is supposed that a part of 5-HT released is taken up again into the synaptosomes by the membranal transport mechanism. The finding that the effect of H 102/09 increased with time is in accordance with this interpretation, since the medium concentration of 5-HT increased and accordingly the uptake velocity The antagonism of the evoked efflux is,

on the other hand, understood, if the transport of 5-HT under these experimental conditions occurs with the reversed uptake mechanism and this efflux accordingly inhibited by the uptake inhibitors

There are several observations supporting the hypothesis that the membranal 5-HT transport mechanism can be reversed The Na dependence of the influx and efflux of 5-HT (Bogdanski *et al* 1968) indicates this possibility since several other Na dependent transport mechanisms are known to be reversible (Schultz and Curran 1970). Na influences these transports either by increasing the affinity of the solute for the uptake sites or by increasing the velocity of the transport Observation in the present study indicates that the affinity of 5-HT is increased by Na which is in variance with the transport systems for noradrenaline (Sugrue and Shore 1971) and dopamine (Holz and Coyle 1974) in which the velocity of the transport but not the affinity appears to be increased The explanation of the efflux of 5-HT by low external Na may accordingly be due to an equalization of the affinities on the two sides of the neurone membrane, which because of the much larger 5-HT concentration inside the synaptosomes results in the outward transport Ouabain by inhibiting the Na pump increase the Na concentration in the synaptosomes and thereby the affinity of the 5-HT for the carrier sites on the inside of the membrane. Veratridine increases the Na permeability of excitable membranes (Ulbricht 1969) with the same result as with ouabain but with a more rapid onset of action The finding that the local anesthetic agent millicaine discriminated between the 5-HT efflux induced by veratridine and low external Na indicates that the membrane stabilizing effect of the test compounds was not involved in the inhibition of the 5-HT efflux mechanism. Thus, the effect by millicaine on the veratridine evoked efflux could be explained by the antagonism of the depolarizing effect whereas the corresponding effect by the uptake inhibitors was probably due to the inhibition of the 5-HT transport In accordance with this view a comparatively high concentration of desipramine, which is a rather poor inhibitor of the 5-HT uptake (Ross and Renyi 1975), had larger effect on the veratridine evoked efflux, reflecting the membrane stabilizing action at this concentration Indeed inhibition of the release of neurotransmitters induced by veratridine in synaptosomal preparations may become a valuable method for testing drugs on the membrane stabilizing action under identical or similar *in vitro* conditions as used in other assays. However the test compound must not inhibit the active outward transport of the transmitter examined

The efflux induced by 4-chloroamphetamine can also be explained by the hypothesis of the reversed transport mechanism. This amine has high affinity for 5-HT neurones (Pletscher *et al* 1964 Fuller *et al* 1965) Since the vesicular storage mechanism of 5-HT was destroyed by reserpine, 4-chloroamphetamine, which because of its large lipophility readily passes membrane barriers, probably released H 5-HT bound to some extravesicular binding sites in the nerve endings. The rise in the concentration of the soluble fraction of 5-HT in the synaptomes results in increase in the outward transport of 5-HT since the rate of the transport is directly related to the concentration of 5-HT within the ranges of the transport capacity The inhibition by the 5-HT uptake inhibitors of the efflux evoked by 4-chloroamphetamine, low external Na and high internal Na at the same concentration ranges support this hypothesis. It can be argued that the uptake inhibitors antagonized

an active transport of 4-chloroamphetamine into the synaptosomes and thereby preventing the intraneuronal release of ^{3}H 5-HT However the finding that ouabain did not inhibit the effect of 4-chloroamphetamine but instead added its own effect to that of 4-chloroamphetamine indicates that 4-chloroamphetamine was not transported by the membranal 5-HT transfer system. Experiments with purified synaptosomes have also shown that ^{3}H 4-chloroamphetamine has high affinity for the synaptosomes but no active, Na$^+$ dependent accumulation was observed (Ross 1976 b).

The inhibition by 4-chloroamphetamine of the 5-HT uptake in the synaptosomes may not in fact be due to a real inhibition of the uptake but due to its 5-HT releasing effect. If the hypothesis of a reversible transport mechanism is correct the influx of 5-HT should equilibrate with the efflux at a much lower ratio between the inner and outer concentration of 5-HT than under normal condition, if the extravesicular binding sites are occupied by 4-chloroamphetamine, which results in an elevated concentration of the free concentration of 5-HT This hypothetical effect of 4-chloroamphetamine should accordingly be very similar to an inhibition of the uptake although the primary attack is not located to the transfer sites This suggestion is in accordance with the observation that 4-chloroamphetamine was more or at least as potent in increasing the efflux than in inhibiting the uptake of 5-HT According to the kinetic experiments H 102/09 inhibited competitively the increased efflux produced by 4-chloroamphetamine. Since the release by 4-chloroamphetamine of intraneuronally bound ^{3}H 5-HT was probably directly related to the concentration of 4-chloroamphetamine the observed competitive nature of the effect of H 102/09 is not contradictory to the hypothesis that H 102/09 inhibited the outward transport of 5-HT The almost identical K_i values for H 102/09 in inhibiting the 5-HT uptake and the 4-chloroamphetamine induced 5-HT efflux further support the hypothesis of an identical mechanism for the uptake of 5-HT and the efflux of 5-HT evoked by 4-chloroamphetamine.

Alternative explanations of the data are possible. For instance, it can not be excluded that the influx and efflux are two separate mechanisms and that the inhibitors of the 5-HT uptake also antagonized the efflux mechanism due to similar structure requirements for both mechanisms.

References

BLASZKOWSKI, T P and D F BOGDANSKI, Evidence for sodium dependent outward transport of ^{3}H-norepinephrine mobilized by calcium at the adrenergic synapse Inhibition of transport by desipramine. *Life Sci* 1972 *11* 867–876

BOGDANSKI, D F A TISSARI and B B BRODIE, Role of sodium, potassium, ouabain and reserpine in uptake, storage and metabolism of biogenic amines in synaptosomes *Life Sci* 1968 *7* 419–428.

FULLER, R W C W HINES and J MILLS, Lowering of brain serotonin level by chloroamphetamines. *Biochem Pharmacol* 1965 *14* 483–488

FURCHGOTT R F S M KIRPEKAR, M. RIEKER and A. SCHWAB, Actions and interactions of norepinephrine, tyramine and cocaine on aortic strips of rabbit and left atria of guinea-pig and cat. *J Pharmacol exp Ther* 1963 *142* 39–58.

HOLZ, R W and J T COYLE, The effects of various salts, temperature, and the alkaloids veratridine and batrachotoxin on the uptake of [^{3}H]-dopamine into synaptosomes from rat striatum. *Molecul Pharmacol.* 1974 *10* 746–758

LINEWEAVER, H and D BURK, The determination of enzyme dissociation constants. *J Amer Chem Soc.* 1934 *56* 658–666

on the other hand, understood if the transport of 5-HT under these experimental conditions occurs with the reversed uptake mechanism and this efflux accordingly inhibited by the uptake inhibitors.

There are several observations supporting the hypothesis that the membranal 5-HT transport mechanism can be reversed The Na dependence of the influx and efflux of 5-HT (Bogdanski *et al* 1968) indicates this possibility since several other Na dependent transport mechanisms are known to be reversible (Schultz and Curran 1970) Na influences these transports either by increasing the affinity of the solute for the uptake sites or by increasing the velocity of the transport Observation in the present study indicates that the affinity of 5 HT is increased by Na which is in variance with the transport systems for noradrenaline (Sugrue and Shore 1971) and dopamine (Holz and Coyle 1974) in which the velocity of the transport but not the affinity appears to be increased. The explanation of the efflux of 5-HT by low external Na may accordingly be due to an equalization of the affinities on the two sides of the neurone membrane, which because of the much larger 5-HT concentration inside the synaptosomes results in the outward transport Ouabain by inhibiting the Na pump increase the Na concentration in the synaptosomes and thereby the affinity of the 5-HT for the carrier sites on the inside of the membrane. Veratridine increases the Na permeability of excitable membranes (Ulbricht 1969) with the same result as with ouabain but with a more rapid onset of action The finding that the local anesthetic agent millicaine discriminated between the 5-HT efflux induced by veratridine and low external Na indicates that the membrane stabilizing effect of the test compounds was not involved in the inhibition of the 5-HT efflux mechanism. Thus, the effect by millicaine on the veratridine evoked efflux could be explained by the antagonism of the depolarizing effect whereas the corresponding effect by the uptake inhibitors was probably due to the inhibition of the 5-HT transport. In accordance with this view a comparatively high concentration of desipramine, which is a rather poor inhibitor of the 5-HT uptake (Ross and Renyi 1975) had larger effect on the veratridine evoked efflux reflecting the membrane stabilizing action at this concentration Indeed inhibition of the release of neurotransmitters induced by veratridine in synaptosomal preparations may become a valuable method for testing drugs on the membrane stabilizing action under identical or similar *in vitro* conditions as used in other assays. However the test compound must not inhibit the active outward transport of the transmitter examined.

The efflux induced by 4-chloroamphetamine can also be explained by the hypothesis of the reversed transport mechanism This amine has high affinity for 5-HT neurones (Pletscher *et al* 1964 Fuller *et al* 1965) Since the vesicular storage mechanism of 5-HT was destroyed by reserpine, 4-chloroamphetamine, which because of its large lipophility readily passes membrane barriers, probably released H 5-HT bound to some extravesicular binding sites in the nerve endings. The rise in the concentration of the soluble fraction of 5-HT in the synaptomes results in increase in the outward transport of 5-HT since the rate of the transport is directly related to the concentration of 5-HT within the ranges of the transport capacity The inhibition by the 5-HT uptake inhibitors of the efflux evoked by 4-chloroamphetamine, low external Na and high internal Na at the same concentration ranges support this hypothesis. It can be argued that the uptake inhibitors antagonized

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Preferential Uptake of ¹⁴C-α Aminoisobutyric Acid into Mouse Uterine Tissue during Early Pregnancy

By

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Abstract

LINDQVIST, I., O. NILSSON and G. RONQUIST. *Preferential uptake of* ¹⁴*C-α-aminoisobutyric acid into mouse uterine tissue during early pregnancy.* Acta physiol. scand. 1977. 99. 37–41.

The uptake of ^{14}C-AIB into uterine tissue and some other organs was studied during early implantation. Mice in experimentally delayed implantation were given estrogen and ^{14}C-AIB at different time intervals. Mice in delayed implantation given only ^{14}C-AIB displayed a low transport rate into the uterine tissue, comparable with that for the diaphragm muscle. However, if the estrogen injection preceded that of the ^{14}C-AIB by 4 to 8 h the transport capacity increased by a factor of at least 4 times, while the uptake for the diaphragm muscle remained low. Under such conditions ^{14}C-AIB-accumulation into uterine tissue was favoured for at least 4 h of in vivo incubation with the labeled amino acid.

The rate of amino acid transport appears to be closely correlated to the rate of protein synthesis of both normal and malignant tissues (Riggs 1964, Griffiths 1967). Glucocorticoids, which are catabolic hormones, work antagonistically to the transport of amino acids into skeletal muscle (Riggs 1964, Christensen 1975), while insulin and growth hormone, for example, which are both anabolic hormones and promote protein synthesis accelerate amino acid transport into several tissues (Riggs 1964). Also estrogens promote growth of particular tissues (Noall *et al.* 1957, Noall and Allen 1961) and consequently McCorquodale and Mueller (1958) observed an increased activity of amino acid-activating enzyme in rat uterus already 3 h after treatment with estrogen. An early "induced protein" after exposure of rat uterus to estradiol, has recently been detected among the soluble proteins (Pennequin, Robel and Baulieu 1975).

The capacity to transport amino acids is probably also higher since the level of free amino acid is higher in the more rapidly growing tissues (Noall *et al.* 1958). This is tested by using α-aminoisobutyric acid, AIB, as an amino acid substrate since this amino acid is not metabolized. As expected, Noall *et al.* (1958) found a 3-fold increase in the uterine accumulation of AIB upon administration of estradiol, and Riggs and Pan (1972) observed an increased

PATON D M Mechanism of efflux of noradrenaline from adrenergic nerves in rabbit atria. *Brit J Pharmacol* 1973 *49* 614–627

PLETSCHER, A., G BARTHOLINI, H BRUDERER, W P BURKARD and K F GEY Chlorinated arylalkylamines affecting the cerebral metabolism of 5-hydroxytryptamine *J Pharmacol exp Ther* 1964. *145* 344–350.

ROSS, S. B., Structural requirements for uptake into catecholamine neurones. In *The mechanisms of neuronal and extraneuronal transport of catecholamines.* Ed D M Paton, 1976 a, pp. 67–93 Raven Press New York.

ROSS, S. B Antagonism of the acute and long-term biochemical effects of 4-chloramphetamine on the 5-HT neurones in the rat brain by inhibitors of the 5-hydroxytryptamine uptake. *Acta pharmacol* (Kbh) 1976 b. *39* 456–476.

ROSS, S. B. and A. L. Renyi, Inhibition of the upt ke of tritiated 5-hydroxytryptamine in brain tissue *Europ J Pharmacol.* 1969 *7* 70–?77

ROSS, S. B and A. L. RENYI, Uptake and metabolism of β-phenylethylamine and tyramine in mouse brain and heart slices. *J Pharm Pharmacol* 1971 *23* 276–79

ROSS, S. B. and A L. RENYI, Tricyclic antidepressa t agents. I Comparison of the upt ke of H-noradrenaline and ^{14}C 5-hydroxytryptamine in slices and crude synaptosome preparations of the midbrain-hypothalamus region of the rat brain. *A ta pharmacol* (Kbh.) 1975 *36* 382 394

ROSS, S. B A. L. RENYI and B BRUNFELTER, Cocaine sensiti e uptake of sympathomimetic amines in nerve tissue. *J Pharm Pharmacol* 1968 *20* 283 ,88.

ROSS, S. B. and D KELDER, Active transport of H-bretylium in the rat vas deferens in itro *Act ph ol scand* 1976. *97* 209 221

ROSS, S. B., S.-O ÖGREN and A. L. RENYI, (Z)-Dimethylamino-1-(4-bromophenyl)-1-(3-pyridyl)propene (H 102/09) and new selective inhibitor of the neuronal 5-hydroxytryptamine uptake *A ta pharmacol* (Kbh) 1976. *39* 152 166.

SCHULTZ, S. G and P F CURRAN, Coupled transport of sodium and organic solutes *Ph siol Rev* 1970 *50* 637 718

SHASKAN, E. a d S. H SNYDER, Kinetics of ^{3}H 5-hydro ytryptamine uptake into synaptosomes f om different regions f the brain. *J Pharmacol xp Ther* 1970 *175* 404–418

SNYDER, S. H and J T COYLE Regional differences in H norepinephrine and H^{3}-dopamine uptake into rat brai homogenates. *J Pharmacol xp Ther 1969 165* 78–86.

SUG UE, M F nd P A SHORE, Further evidence for a sodium-dependent, optically specific nd reserpine-sensitive amine carrier mechanism at the adrenergic neuron. *J Pharmacol xp. Ther* 1971 *177* 389 397

THOA, N B G F WOOTEN, J AXELROD and I J KOPIN, On the mechanism of release of norepinephrine from sympathetic nerves induced by depolarizing agents and sympathomimetic drugs. *Mol Pharmacol* 1975 *11* 10–18

TRENDELENBURG, U Classification of sympathomimetic amines. In *Catecholamines.* Ed. H Blaschko *and E. Muscholl, 1972, pp* 336–362. Springer Verlag Berlin, Heidelberg, New York

ULBRICHT W The effect of veratridine on excitable membranes of nerve and muscle *Ergebn Physiol.* 1969 *61* 18 71

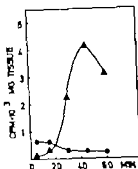


Fig. 1

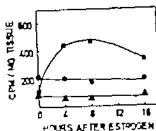


Fig. 2

Fig 1 ^{14}C AIB concentration in blood and recovery in uterus at different times after intravenous administration ●—● blood, ▲—▲ uterus.

Fig 2 Recovery of ^{14}C-AIB in blood (●—●), diaphragm muscle (▲—▲) and uterine tissue (●—●) at different stages of implantation initiated by estrogen. Stand. dev. max ± 40 cpm.

mice of the other 3 groups were given an injection of estrogen, and ^{14}C AIB was administered 20 min before death, 4, 8 and 16 h respectively after the estrogen injection. Blood, diaphragm muscle and uterus were examined.

The level of ^{14}C-AIB in the blood was about the same between 0 and 16 h after the injection of estrogen. The *in vivo* uptake by the diaphragm muscle was quite low and independent of the time after the estrogen injection. The *in vivo* uptake by the uterine tissue, on the contrary, displayed a characteristic profile with a low uptake of ^{14}C AIB for animals in delayed implantation (controls), marked increase at 4 and 8 h after the initiation of implantation by estrogen and a slight decrease at 16 h as compared with the values for 4 and 8 h (Fig. 2).

The retention of ^{14}C-AIB was measured in blood, diaphragm muscle, brain and uterus. Mice were given the labeled amino acid 4 h after the injection of estrogen and the tissue concentrations were measured after different *in vivo* incubation times with the labeled amino acid substrate. 5 groups, each containing at least 3 animals in experimentally delayed implantation were used. The animals were sacrificed 1/3, 4, 16, 24 and 48 h after the injection of ^{14}C AIB (Fig. 3).

Although the blood concentration of the labeled amino acid was declining already at 20 min after the i.v. administration (*cf.* Fig. 1) and remained low throughout the expt., the ^{14}C-AIB was retained in the uterus for several hours. Still at 16 h the uterine concentration of the labeled amino acid was higher than the 20 min blood value. Also after 16 h the blood value was 7 times lower than that of the uterus. Diaphragm muscle showed a slow accumulation of ^{14}C AIB during at least the first 4 h and retained the labeled amino acid for several hours. The brain displayed a somewhat longer accumulation time and a higher uptake than the diaphragm muscle. The uptake of ^{14}C AIB was nevertheless much lower in these tissues than in the uterus. The prolonged uptake process into brain tissue *in vivo* might be explained by the existence of specific carrier systems for amino acids in the blood-brain barrier (Oldendorf 1973) with a relatively slow transport rate for AIB (Oldendorf 1971).

in vitro transport of AIB into the estrogen primed uterus. Other studies also indicate an influence *in vitro* by estrogen on AIB uptake and transport (Riggs *et al* 1968, Walters *et al* 1975)

Since early pregnancy specifically the time of blastocyst implantation, is associated with an increase in the concentration of estrogen we questioned whether the capacity of AIB transport is increased as well If so this might have implications for the nutrition of the blastocyst at trophoblast attachment and invasion Therefore, the aim of the present investigation was, firstly to examine the characteristics of AIB transport *in vivo* into some organs of mice, conditioned for experimentally delayed implantation (Humphrey 1967) and secondly to examine whether the capacity of AIB transport *in vivo* into the uterus and some other organs increased after the initiation of implantation by estrogen

Materials and methods

¹⁴C-α-aminoisobutyric acid (1 ¹⁴C AIB), Protosol and Aquasol were purchased from NEN Chemicals GmbH Dreieichenhain, W Germany and α-aminoisobutyric acid from Calbiochem, San Diego, USA Progesteron was purchased from Ikapharm, Ramat-Gan, Israel and estradiol from AB Leo Hälsingborg, Sweden All chemicals used were analytical grade Radioactivity was measured in a Nuclear Chicago Unilux II Liquid Scintillation Counter

Adult white female mice (N M R.I. Naval Medical Res. Inst., Bethesda, USA) were mated and the day the vaginal plug was seen was recorded as day 1 They were spayed on day 3 and given the first dose of 1 mg of progesteron in 0 04 ml peanut oil subcutaneously the same day The animals were kept in delayed implantation by daily injections of progesteron Implantation was initiated by a subcutaneous injection of 0 1 g estradiol 17B in 0 1 ml propyleneglycol. Generally 4.25 μCi ¹⁴C AIB in 0.1 ml of a 40 mM AIB solution, made isotonic with NaCl, was given i v in the tail to each animal. Blood samples were drawn from the cut tail and directly transferred to the liquid scintillation bottles. Tissue samples were washed once in ice-cold Krebs-Ringer bicarbonat buffer freshly bubbled with a gas mixture of 93.5 O_2 and 6 5 CO_2 to give a pH of 7 4 and lightly blotted on tissue paper before being transferred to the bottles The animals were sacrificed by giving them Nembutal before the tissue samples were taken.

Samples were transferred to weighed liquid scintillation counting bottles, weighed and dissolved over night in 1 ml Protosol at 50°C. The solutions were mixed with 50 μl conc. acetic acid (to minimize [illegible] sensitivity) and 10 ml of Aquasol Samples were then counted and countings corrected for self absorption.

Results

The clearance of ¹⁴C AIB was established by determining the time at which the amino acid concentration curve of the blood crossed that of the urine The labeled amino acid was i v administered to the animals which were then sacrifized at different times between 5 and 20 min after the injection of the labeled amino acid. Fig. 1 shows that a peak value in blood was reached after 15 min and that the declining blood curve crossed the rising curve reflecting the urine concentration of ¹⁴C AIB after 20 min The curve profiles of both blood and urine were quite similar in 4 different expts. with animals in delayed implantation as well as with estrogen-treated animals. It was therefore convenient to sacrifize the animals 20 min after the i v administration of ¹⁴C-AIB during the next series of expts.

The distribution of ¹⁴C AIB into the uterus and some other tissues during early implantation was examined in 4 groups, each containing 3 mice in experimentally delayed implantation. Animals of one group (controls) were given only ¹⁴C AIB 20 min before

During activation for implantation the first 8 h are a period when system A amino acids are supplied from the blood into the uterine tissue at a high degree. Perhaps this accumulation of amino acids in the uterine tissue can be exploited also by the developing blastocyst. If so, AIB should accumulate also [illegible] the blastocysts. This possibility is presently being tested.

This investigation was supported by grants from the Swedish Medical Research Council, project no B76-13X 228-LLA and no 12X 70. Technical assistant [illegible] as Mrs Barbro Einarsson.

References

Christensen, H. N. *Biological Transport*, 2nd ed. 1975. W. A. Benjamin, Inc., Reading, Mass. U.S.A.

Christensen, H. N. and M. E. Handlogten, Modes of Mediated Exodus of Amino Acids from the Ehrlich Ascites Tumor Cells. *J. biol. Chem.* 1968. *243*. 5428–5438.

Griffiths, J. B. and S. J. Pirt, The uptake of amino acids by mouse cells (strain LS) during growth in batch culture and chemostat culture: the influence of cell growth rate. *Proc. roy. Soc. B*. 1967. *168*. 421–438.

Humphrey, K. W. The induction of implantation in the mouse after ovariectomy. *Steroids* 1967. *10*. 591–601.

McCorquodale, D. J. and G. E. Mueller, Effect of estradiol on the level of amino acid-activating enzymes in the rat uterus. *J. biol. Chem.* 1958. *232*. 31–42.

Noall, M. W. and W. M. Allen, Early stimulation by estradiol of amino acid penetration in rabbit uterus. *J. biol. Chem.* 1961. *236*. 2987–2990.

Noall, M. W., T. R. Riggs, L. M. Walker and H. N. Christensen, Endocrine control of amino acid transfer. *Science* 1957. *126*. 1002–1005.

Oldendorf, W. H. Brain uptake of radiolabeled amino acids, amines and hexoses after arterial injection. *Amer. J. Physiol.* 1971. *221*. 1629–1639.

Oldendorf, W. H. Stereospecificity of blood-brain barrier permeability to amino acids. *Amer. J. Physiol.* 1973. *224*. 967–969.

Oxender, D. L. and H. N. Christensen, Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cell. *J. biol. Chem.* 1963. *238*. 3686–3699.

Peeters, P. P. Rombauts and E.-E. Baulieu, Steroid-induced early protein synthesis in rat uterus and prostate. *Europ. J. Biochem.* 1975. *60*. 137–145.

Riggs, T. R. *Action of hormones on molecular processes*. Eds. Litwack, G. and D. Kritchevsky. Wiley & Sons Inc. New York 1964. 1–57.

Riggs, T. R. and M. W. Pan, Transport of amino acids into the oestrogen primed uterus. Enhancement of the uptake by [illegible] preliminary incubation. *Biochem. J.* 1972. *128*. 19–27.

Riggs, T. R., M. W. Pan and H. W. Feng, Transport of amino acids into the estrogen primed uterus. *Biochim. biophys. Acta* (Amst.) 1968. *150*. 92–103.

Riggs, T. R., M. W. Pan and H. W. Feng, Transport of amino acids into the estrogen primed uterus. III. Effect of the level of extracellular sodium ion. *J. biol. Chem.* 1972. *247*. 7128–7134.

Walters, M. R., A. L. Lawrence and R. L. Hazelwood, Amino acid transport by the rat uterus during the estrous cycle and pregnancy. *Biol. of Reprod.* 1975. *12*. 383–388.

Webber, W. A. A comparison of the efflux rates of AIB from kidney cortex slices of mature and newborn rats. *Canad. J. Physiol. Pharmacol.* 1968. *46*. 765–769.

Webber, W. A. and J. A. Cairns, A comparison of the amino acid concentrating ability of the kidney cortex of newborn and mature rats. *Canad. J. Physiol. Pharmacol.* 1968. *46*. 165–169.

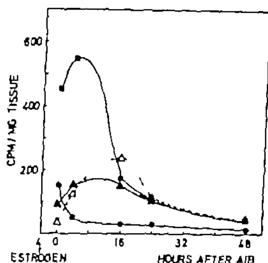


Fig. 3 Retention time of ^{14}C AIB in blood (o—●) diaphragm muscle (—), brain (Δ—Δ) and uterine tissue (■—■). ^{14}C AIB was administrated 4 h after estrogen initiation of implantation Stand. dev = max ± 50 cpm.

Discussion

Mice in delayed implantation which in addition to progesteron had received estrogen or 8 h before being given ^{14}C AIB i v displayed a high uterine uptake compared with mi not having received estrogen Furthermore this uptake was preferential for the uterine tissu compared with some other tissues. Since the ^{14}C AIB transport was considerably low when only ^{14}C AIB was given pretreatment *in vivo* with estrogen is necessary for the enhance ^{14}C AIB-accumulation This is in agreement with the findings of Riggs *et al* (1968) fo the *in vitro* uptake of AIB in immature rat uterus after *in vivo* preincubation with estradio Since the estrogen effect is not momentanous it seems probable that the hormone migh induce a *de novo* synthesis of AIB-carriers in the plasma membranes of uterine tissue. Suc a proposal can be further supported by the determination of the V_{max} parameter and Rigg *et al* (1968) have shown that in their experimental system V_{max} increased while K_m remaine unchanged by the treatment with estradiol

AIB has been shown to be transported mainly by the route defined as the system A by Oxender and Christensen (1963) Several other amino acids as alanine, methionine and nor leucine are mainly transported by this route in different cells. Characteristical features fo this system are the Na -dependency and transport against a concentration gradient The means necessarily that the exit rate by this system is slower than the entrance rate (Christenser and Handlogten 1968) and that the carrier operates asymetrically By this system it is therefore possible to augment the free intracellular amino acid pool while an exchange system can only influence the qualitative composition of the intracellular amino acid pool without enhancing the net uptake. Such an asymmetry has been shown to exist in slices of newborn rat kidney cortex while absent in adult cortex (Webber 1968 Webber and Cairns 1968)

The strict Na -dependence of the AIB-uptake into uterine tissue can naturally not be demonstrated by *in vivo* expts. However Riggs *et al* (1972) have shown that the uptake of AIB into the estrogen primed rat uterus *in vitro* increased regularly with increasing Na amounts in the incubation buffer Thus we have reasons to believe that the system A is

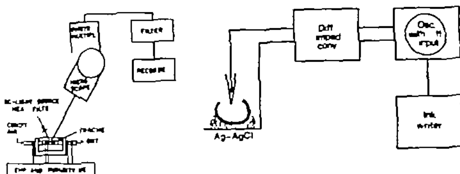


Fig. 1. Block diagram of the experimental equipment for recording mucociliary wave frequency (left) and intracellular activity (right).

lower temperatures is due to physical properties of the secretion layer and not to the intracellular "pace-maker" mechanism. However this conclusion was drawn from recordings of the intracellular and extracellular activities in separate specimens. Simultaneous recordings from the same area would be even more valuable for theoretical and practical purposes. The aim of the present investigation has therefore been 1) to device a method for the simultaneous recording of the intracellular electrical activity and the extracellular mucociliary wave frequency in the same area on tracheal specimens and 2) to compare the frequencies of the electrical discharge and the mucociliary movements at different temperature levels within the range of 20–40 C

Methods and material

Rabbits weighing 1.5–2.0 kg have been used as experimental animals. To avoid undesired pharmacological side effects they have been sacrificed by a blow on their heads. After a careful dissection of the trachea a 3.5–4.0 cm specimen has been removed and put into an experimental chamber in which temperature and relative humidity can be regulated and recorded.

Recording of the mucociliary activity

The recording technique has already been extensively described (Mercke *et al.* 1974). Shortly a cold light beam is directed against the tracheal ciliated mucous membrane through a slit in the upwards directed pars membranacea. Variations of the light intensity of the mucosal reflex are picked up by a microscope (Fig. 1). A photomultiplier (EMI 9524 B) attached to one of the oculars converts the light intensity variations to electrical signals which after amplification and filtering (Krohn Hite type 3550) are recorded by an inkwriter (Elema Mingograph 34).

Recording of the intracellular electrical activity

The technique of recording is a modification of the method originally described by Håkansson and Toremalm in 1966 and has in detail been described by Toremalm *et al.* (1975). In the actual investigation a further improvement has been introduced by exchanging the formerly used transistorized impedance converter to a low frequency impedance converter with a resistance of 1 000 MΩ and an amplification of 1:1 (Fig. 1).

Experimental procedure

After the tracheal specimen has been placed into the experimental chamber, the capillary microelectrode is very slowly pushed downwards through the mucus into the cell layer in the center of the light reflex. The oscilloscope is then in the DC position. When a potential change of -15 to -40 mV indicates that a ciliated

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Intra and Extracellular Activity of Ciliated Cells

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Abstract

Huberman D C H Håkansson U Mercke and N G Toremalm *Intra- and extracellul activity of ciliated cells* Acta physiol scand 1977 99 42–47

A method permitting simultaneous recording *in vitro* of the intracellular and extracellula (= muco ciliary) activity of respirat ry ciliated mucous membrane is presented. Using this method the influenc of different temperature levels (20 30° 40°C) upon the tracheal mucous membrane of 15 rabbit has bee investigated. It is show 1) that the extracellula (= mucociliary) activity increases with rising temperatur whereas the intracellular activity is unaffected and remains constant, indicat g a temperature influenc upo the respiratory tract mucus rather than a cha ge of the intracellular pace-maker mechanism) that the restraini g effect of the mucus decreases with rising temperatures but is st ll 10 of the intra cellular frequency t body temperature 3) that the presented method should be useful for f t re investi ti tions concerning the effect of air-poll tion nd pharmacological substances upon respiratory mucou membranes.

K y ords. Trachea, mucous membranes, ciliated cells, mucociliary activity elect ophysiological activit

The mechanical defence mechanism of the respiratory mucous membrane is constituted of two factors—the propulsive movements of the cilia and the more or less restricting effect of the covering layer of mucus. The term mucociliary activity has therefore been found more adequate than the previously used designation, "the ciliary beat frequency (Mercke *et al* 1974 a) It has hitherto not been possible to separate the rhythm and frequency of intrinsic ciliary activity from that of the extrinsic mucociliary activity observed through a microscope, but such a functional difference should be of great practical importance for experimental and diagnostic purposes. However this is not possible with the use of extracellular recording methods only A combination of intra- and extracellular measurements is necessary

The intracellular electrical activity in beating ciliated cells was first recorded in vertebrates by Håkansson and Toremalm in 1966. In recent experimental studies we found that the frequency of intracellular action potentials was about the same at 20 30 and 40 C during *in vitro* conditions while the extracellular movements of the cilia decreased considerably with decreasing temperatures (Toremalm *et al* 1975). This means that the frequency change

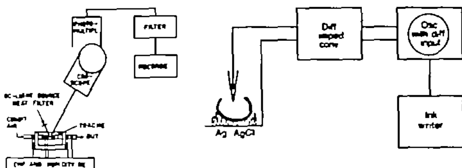


Fig 1 Block diagram of the experimental equipment for recording mucociliary wave frequency (left) and intracellular activity (right).

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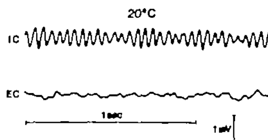


Fig. 2. Recordings from one tracheal specimen showing the intracellular activity (IC) and the mucocillary wave pattern (EC) at 20°C.

cell membrane has been penetrated, the oscilloscope is changed to the AC position and the above described intracellular recording is commenced. The microscope is then adjusted so that variations of the light reflections exactly corresponding to the area of intracellular recording may be picked up and recorded.

The simultaneous recording of intracellular electrical and extracellular mechanical activities from the very small mucosal area has been performed at 3 different temperature levels (20, 30 and 40°C) and at an environmental air humidity above 90%. The trachea from 5 rabbits have been investigated at each temperature level.

Results

At 20°C (Fig. 2) the intracellular potential recordings show a fairly even rhythm and a frequency of 20 cycles/s whereas the extracellular recordings are irregular with a low frequency of about 12 waves/s (Table I).

At 30°C (Fig. 3) the intracellular frequency does not change in contrast to the extracellular activity which has increased. The difference in the intra- and extracellular activities is

TABLE I

Temp. (°C)	Intracellular activity (oscillations/s)	Extracellular (mucocillary) activity (waves/s)
20	20.5	9.6
	21.5	13.1
	21.0	17.1
	21.7	10.4
	21.3	12.7
	M: 21.2	M: 12.6
30	21.3	16.7
	21.5	17.4
	23.1	18.0
	21.4	17.6
	20.8	16.3
	M: 21.6	M: 17.2
40	22.1	18.8
	22.0	19.5
	21.5	17.7
	21.2	18.8
	21.5	17.4
	M: 21.5	M: 18.4

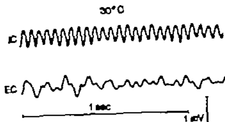


Fig. 3. Recordings from one tracheal specimen showing the intracellular activity (IC) and the mucociliary wave pattern (EC) at 30°C.

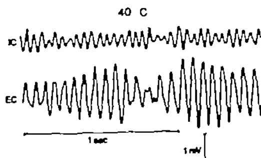


Fig. 4. Recordings from one tracheal specimen showing the intracellular activity (IC) and the mucociliary wave pattern (EC) at 40°C.

markedly reduced (20 cycles and 17 waves/s respectively Table I). At 40°C (Fig. 4) this difference is even more reduced, now being 20 cycles and 18 waves/s respectively (Table I).

The frequency/temperature relationship concerning the intracellular as well as the extracellular recordings is illustrated in Fig. 5. The plotted values are the mean frequencies of all 5 rabbit specimens at each temperature level (Table I). From this figure it is clearly evident that the intracellular activity is mainly unaffected by temperature changes in the interval 20°C to 40°C. However the extracellular or mucociliary activity increases almost linearly with increasing temperature in the same interval.

Discussion

A comparative study of the intracellular electrical activity which initiates the movements of the cilia covering the surface of the respiratory tract, and the extracellular manifestations

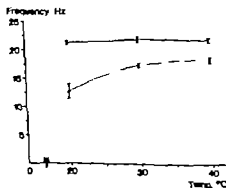


Fig. 5. The frequency/temperature relationship regarding intracellular (broken line) and extracellular (dotted line) activities simultaneously monitored. Each plotted value is the average of 5 measurements.

of this activity is presented in this investigation The experimental model is a combination of two previously described methods (Fig. 1) dealing with one of these activities at a time (Mercke *et al* 1974 a, Toremalm *et al* 1975) It has been possible to make simultaneous recordings of the intracellular electrical activity in one ciliated cell and the extracellular mechanical activity from a small area covering about 10 ciliated cells during standardized conditions regarding temperature and humidity of the surrounding air

This investigation has been limited to a comparison of the two mentioned activities at three different temperature levels. Typical recordings at 20° 30° and 40 °C are demonstrated in Fig. 2, 3 and 4 respectively The entire results regarding all tracheal specimens are shown in Table I and are also graphically illustrated in Fig. 5

The relative constancy of the intracellular oscillations from 20 to 40°C is a physiologically interesting phenomenon. In fact this may have two explanations 1 The rhythm of the "pace-maker is relatively insensible to temperature changes. 2. The frequency may be constant but the rising time of the potential fluctuation can be shorter and the rise more rapid at higher temperatures. This may result in an increasing amplitude of the intracellular recording. Such an explanation cannot be deduced from Fig. 2, 3 and 4 since the recordings at 20 30 and 40°C are obtained from different tracheal specimens. To be able to answer the hypothesis furthe expts. have to be done where one tracheal specimen is investigated at the different temperatures.

The extracellular mucociliary activity on the other hand is strongly influenced by temperature changes. This has been described more in detail in two previous papers (Mercke *et al* 1974 b Mercke 1974) From the present simultaneous recordings frequency differences between the intra and extracellular activities increase by decreasing temperature levels below 40 C (Fig. 5) Ordinary body temperature therefore seems to be optimal for the cilia to work in. In other words the mechanical restriction of the secretion layer is minimal at this temperature level However even at optimal temperature conditions there is a difference of about 2 cycles/s between the internal and external activities. It means that the secretion layer normally has a decelerating effect upon the movements of cilia. The consequences of the present results are (1) that the method makes it possible to separate the intracellular ciliary activity (the "pace maker" effect) from the retarding effect of the mucus layer surrounding the cilia, and (2) that this inhibitory factor reduces the frequency by about 10 at and above body temperature.

This knowledge seems to be valuable for future experimental investigations in which the model may be used *e.g* for air pollution tests and control of pharmacological substances for local treatment of the respiratory tract From the results a hypothetical conclusion may be drawn implying that it must be easier to stimulate the mucociliary defence mechanism by changing the physical properties of the mucus than to try to accelerate the intracellular activity of the ciliated cells. However this hypothesis need to be proven by a series of further expts. which are now in progress.

This investigation has been supported by grants from th Swedish Medical Research Council project nr B75-17X 3897-03A.

References

HÅKANSSON, C. H. and N. G. TOREMALM, Studies on the physiology of the trachea. III. Electrical activity of the ciliary cell layer. *Ann. Otol.* (St. Louis) 1966, 75, 1007–1018.

MERCKE, U. The influence of temperature on mucociliary activity. Temperature range 40°C–50°C. *Acta oto-laryng.* (Stockh.) 1974, 78, 253–258.

MERCKE, U., C. H. HÅKANSSON and N. G. TOREMALM, A method for standardized studies of mucociliary activity. *Acta oto-laryng.* (Stockh.) 1974 a, 78, 118–123.

MERCKE, U., C. H. HÅKANSSON and N. G. TOREMALM, The influence of temperature on mucociliary activity. Temperature range 20°C–40°C. *Acta oto-laryng.* (Stockh.) 1974 b, 78, 444–450.

TOREMALM, N. G., C. H. HÅKANSSON, U. MERCKE, B. DALHAMN and D. HYBBINETTE, Mucociliary wave pattern. A comparative analysis of intracellular and extracellular activity. *Acta oto-laryng.* (Stockh.) 1975, 79, 436–441.

Acta physiol. scand. 1977 99 48-52
From the Institute of Physiology University of Lund Sweden

The Effect of Teeth Amputations on the Choline Acetyltransferase Activity of Rat Submaxillary Glands

By

JÖRGEN EKSTRÖM

Received 1 May 1976

Abstract

EKSTRÖM J *The effect of teeth amputations on the choline acetyltransferase activity of rat submaxillary glands.* Acta physiol. scand 1977 99 48-52.

The choline acetyltransferase activity in parasympathetically decentralized glands was unaffected by repeated teeth amputations over a period of 2 weeks, while after the same period of time the treatment caused the enzyme activity to increase in innervated glands. It appears that the enzyme in the postganglionic nerve is for its activity dependent on an intact connection with the central nervous system. The increase in enzyme activity is attributed to an enhanced reflex stimulation of the glands from pulpal receptors.

Key words Choline acetyltransferase activity salivary glands, impulse flow

When the parasympathetic postganglionic neurones of the salivary glands loose their connection with the central nervous system by a section of the preganglionic nerve supply their capacity to synthesize acetylcholine has been found to diminish, as judged from the fall in the activity of choline acetyltransferase (Nordenfelt 1964 Ekstrom and Holmberg 1972). Based on this observation Nordenfelt (1964 1965) suggested that the activity of the acetylcholine forming enzyme might be influenced by the intensity of the impulse traffic in the neurones as to the adrenergic neurones a similar relationship between the activity of catecholamine synthesizing enzymes and impulse traffic has since been considered (Zieher and Pellegrino de Iraldi 1966, Molinoff and Axelrod 1971 Thoenen 1974). The idea put forward by Nordenfelt is favoured by the outcome of various types of experiments on salivary glands, where the degree to which the glands were activated from the central nervous system was either decreased or increased over a long time period (see Ekstrom 1975) In parotid glands of rats the choline acetyltransferase activity declined when the animals were kept on a liquid diet, while it increased when the diet was dry and bulky or when the mouth and the throat of the animals were made dry by atropinization or salivary duct ligations. The increase in the choline acetyltransferase activity in the submaxillary glands of the rat after repeated teeth amputations reported by Ohlin and Perec (1967) seems also to be a finding, which would fit into the pattern outlined above the enzyme activity is increased as a conse-

of the enhanced stimulation of the glands reflexly elicited by the irritation of pulpal receptors. However these authors reported that in the parasympathetically decentralized glands the choline acetyltransferase activity had increased after the amputations. This might suggest that some other factor than the impulse traffic is responsible for the increased enzyme activity seen after teeth amputations.

In the present study the effect of teeth amputations on the choline acetyltransferase activity was compared in decentralized and innervated submaxillary glands.

Methods

50 male rats of Sprague-Dawley strain bred at this Institute were used. They were at the start of the expts. 4–6 months old. The diet used was of gruel-like consistency prepared daily by mixing powder made of standard pelleted diet with water, so as to make easier for the rats with amputated teeth to eat, the rats serving as controls were also given this diet. The diet and water were given *ad libitum*. Both at the start and at the end of the experimental period the body weights of the rats were determined.

In rats lightly anesthetized with ether both the lower and upper incisors were cut to the gingival margin with bone cutting forceps every other day over a period of either 14 or 21 days. The rats serving as controls were also exposed to ether for a few minutes at the same occasion.

In one type of expt the chorda-lingual nerve was bilaterally cut under a dissecting microscope in 18 rats and a few mm of the nerve was removed. The wound was then sutured. In 9 of these rats teeth amputations started 7 days later and continued over a time period of 14 days, the other 9 rats were litter mates to the amputated ones and served as controls. In another type of expt. rats with an intact innervation of their salivary glands were exposed to the amputations. In a first series, the incisors of 9 rats were amputated over a period of 14 days, in a second series 7 rats were amputated over a period of 21 days, 9 and 7 untreated litter mates to those which were amputated served as controls.

At the end of the experimental period the rats were killed by inhalation of ether and the submaxillary glands were removed (the sublingual glands being separated from the submaxillaries), washed in saline, cleaned, pressed gently between gauze pads and weighed. In the rats studied over the period of 3 weeks the sublingual glands were also removed and treated in the same way as the submaxillaries. Both wet and dry weights were determined, the latter expressed as acetone dried powder.

The glands were then prepared for the analysis of their activity of choline acetyltransferase using the method devised by Hebb (see Nordenfelt 1963, 1965). Acetone dried powder was made of the 2 pooled submaxillary glands of one rat, as to the sublingual glands each pool consisted of the glands of 7 rats. The powder of the glands was made up in cysteine-saline in a concentration of 50 mg/ml. Of the tissue extract 0.2 ml was incubated at 38°C for 60 min. Experimental preparation and control preparation were incubated simultaneously and the assay for acetylcholine was made on the same eserinized frog rectus. The choline acetyltransferase activity is expressed in µg acetylcholine chloride formed per h per pooled glands (total activity) and in µg acetylcholine chloride formed per h per g acetone powder (concentration).

Student's t-test was used, paired comparisons were made between the experimental rat and its control litter mate. P values of less than 0.05 were considered significant.

Results

Body weights. At the start of the expts. no differences existed in body weights between the rats to be used as controls and those to be teeth amputated. The body weights of the teeth amputated rats with decentralized glands decreased ($p < 0.001$) during the 14 days from (mean ± S.E.) 294 ± 8 to 259 ± 8 g ($n = 9$), i.e. by 12%. During the same time period the control litter mates increased ($p < 0.001$) in weights by 7%, from 293 ± 9 to 314 ± 9 g ($n = 9$). The body weights of the teeth amputated rats with an intact innervation decreased from 301 ± 7 to 267 ± 8 (9) g, i.e. by 11% in the expt. of the duration of 14 days and in the expt.

TABLE I Effects of repeated teeth amputations on the wet weight and choline acetyltransferase activity of submaxillary glands, parasympathetically decentralized or normally innervated. For the estimation of the enzyme activity the glands from one rat were pooled. Number of observations is given in brackets. Values are mean ± S.E.

Days	Experiments	Wet weights mg	Enzyme activity	
			In μg ACh/h/ pooled glands (total activity)	I μg ACh/h/ g acetone powder (concentration)
14	Decentralization + Amputations	31..4 ± 8 7 (18)[4]	36.7 ± 1 9 (9)	.89 ± 17 (9)[4]
	Decentralization	168 9 ± 4.7 (18)	36.6 ± 2.4 (9)	547 ± 21 (9)
14	Amputations	287 4 ± 8.4 (18)[4]	51 5 ± .9 (9)[4]	452 ± 37 (9)[4]
	No treatment	200.4 ± 2.7 (18)	46.3 ± 3.3 (9)	555 ± 39 (9)
1	Amputations	358.7 ± 22.5 (14)[4]	43 6 ± 2.7 (7)[4]	306 ± .3 (7)[4]
	No treatment	.30.7 ± 4 7 (14)	37 7 ± 1 5 (7)	4 6 ± 18 (7)

[1] >0.1 <0.05. <0.01 [4] <0 001 When the glands of the teeth amputated rat are compared with those of the control litter mate.

of 21 days from 350 ± 10 to 305 ± 9 g (n = 7), i e. by 13% the difference is significant at the $p < 0.001$ and < 0.01 levels respectively In the expt. of 14 days the controls increased ($p < 0.001$) in weights by 12% from 305 ± 6 to 342 ± 6 g (n = 9), while the weights of the controls in the expt of 21 days did not change significantly (from 350 ± 9 to 359 ± 6 g, n = 7)

Gland weights and choline acetyltransferase activity

The effects of teeth amputations on the weights, the total enzyme activity and the enzyme concentration of the submaxillary glands are shown in Table I and in Fig. 1

Decentralized glands The submaxillaries were 96% heavier (dry weights) in the rats exposed

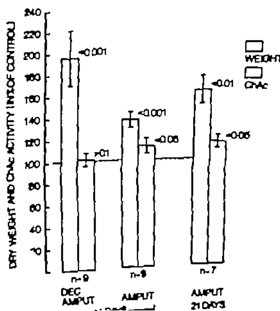


Fig. 1 Dry weight and choline acetyltransferase activity (calculated per pool of 2 glands) of the parasympathetically decentralized or the innervated submaxillary glands after teeth amputations over period of 14 21 days The weight and the enzyme activity are expressed percentage of those of the glands of the control litter mate (mean ± S E.) Number of comparisons and the p-values are given

to teeth amputations than in the rats serving as controls. The total choline acetyltransferase activity did not differ significantly between the glands of the teeth amputated rat and those of the control litter mate. The enzyme concentration was lower in rats after teeth amputations than in controls; this is attributed to the marked gain in weight.

Innervated glands. In the teeth amputated rats the submaxillary glands were 38 and 65% heavier (dry weights) than in the respective controls 14 and 21 days after the beginning of the expt., the difference between the percentage figures was almost significant (0.05 < p < 0.1). The total enzyme activity was 13 and 16% higher in the glands of the teeth amputated rats than in the glands of the control rats after 14 and 21 days, respectively: the percentage figures did not differ from each other significantly. The enzyme concentration was lower than in the controls both after the short and the long experimental period.

When the sublingual glands were examined in the rats exposed to teeth amputations over a period of 21 days the dry weight and the total enzyme activity were 135 and 118% of that of the control glands, respectively.

Discussion

In the present study teeth amputations over a period of 2 weeks were found to have left the total choline acetyltransferase activity unchanged in the parasympathetically decentralized submaxillary glands, while the enzyme activity in the glands supplied with an intact innervation was increased by about 13 per cent after the same period of time. It was not further augmented when the amputations continued for another week. It appears from the present study that the choline acetyltransferase in the postganglionic neurones of the submaxillary glands is for its activity dependent on an intact connection with the central nervous system: the enzyme activity increased in glands normally innervated in response to the teeth amputations, while it was not influenced by the treatment when the preganglionic nerve had been sectioned in advance. It appears reasonable to attribute the small increase in enzyme activity of the submaxillary gland to an enhanced stream of impulses in the parasympathetic nerve reflexly evoked by the activation of pulpal receptors, this is also the likely explanation to the small increase observed in the sublinguals. In this connection it should be pointed out that the percentage increase in enzyme activity found in some other type of expts., in which the reflex stimulation is thought to have been increased, was also moderate, *i.e.* in the range of 10–30 per cent (see Ekström 1975).

There is no ready explanation to the difference between the result obtained in this study and that in the study of Ohlin and Perec (1967), who found in the decentralized glands, enlarged by about the same percentage figure as in the present work and also studied over the same time period, a choline acetyltransferase activity that was twice as high as in the glands of controls. In the two investigations the animals were of the same sex and belonged to the same strain. Of importance for the outcome of the present study may be that comparisons were made between an experimental rat and a control rat of the same age and furthermore, belonging to the same litter. In a recent report attention was drawn to the fact that the choline acetyltransferase activity varies with the age of the animals (Ekström 1976). It

TABLE I Effects of repeated teeth amputations on the wet weight and choline acetyltransferase activi of submaxillary glands, parasympathetically decentralized or normally innervated. For th estimation of the enzyme activity the glands from one rat were pooled. Number of observatio is given in brackets. Values are mean ± S.E.

Days	Experiments	Wet weights mg	Enzyme activity	
			I µg ACh/h/ pooled glands (total activity)	In µg ACh/h/ g acetone powder (concentration)
14	Decentralization + Amputations	312.4 ± 8.7 (18)[4]	36.7 ± 1.9 (9)[1]	289 ± 17 (9)[4]
	Decentralization	168.9 ± 4 7 (18)	36.6 ± .4 (9)	547 ± 23 (9)
14	Amputations	287 4 ± 8.4 (18)[4]	51.5 ± 2.9 (9)[2]	45 ± 37 (9)[3]
	No treatment	200.4 ± 2.7 (18)	46.3 ± 3.3 (9)	555 ± 39 (9)
21	Amputations	358 7 ± 22.5 (14)[4]	43 6 ± 2.7 (7)[3]	306 ± 23 (7)[4]
	No treatment	230.7 ± 4 7 (14)	37 7 ± 1.5 (7)	426 ± 18 (7)

> 0.1 < 0.05 [#] < 0.01 < 0 001 When the glands of the teeth amputated rat are compare with those of the control litter mate

of 21 days from 350 ± 10 to 305 ± 9 g (n = 7) i e. by 13% the difference is significant at th $p < 0.001$ and < 0.01 levels, respectively In the expt of 14 days the controls increase ($p < 0.001$) in weights by 12% from 305 ± 6 to 342 ± 6 g (n = 9) while the weights of th controls in the expt of 21 days did not change significantly (from 350 ± 9 to 359 ± 6 g, n = 7)

Gland weights and choline acetyltransferase activity

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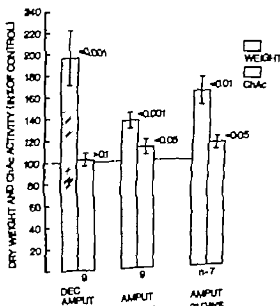


Fig 1 Dry weight and choline acetyltransferase activity (calculated per pool of 2 glands) of the parasympathetically decentralized the ner vated submaxillary glands after teeth mputations over a period of 14 or 21 days The we ght nd the enzyme activity are expressed as percentage of those of the gland of the control litter mate (mean ± S E.). N mber of comparisons nd the p-values are given.

Acta physiol. scand. 1977 99 53–61

From the Department of Medical Pharmacology University of Uppsala, Sweden

Carbonic Anhydrase in the Intestinal Tract of the Guinea-pig

By

GUDMAR LÖNNERHOLM

Received 24 May 1976

Abstract

LÖNNERHOLM, G. *Carbonic anhydrase in the intestinal tract of the guinea-pig.* Acta physiol. scand. 1977 99 53–61

The distribution of carbonic anhydrase activity in the intestinal tract of the guinea-pig was studied by the histochemical method of Hansson. Enzyme activity was demonstrated in epithelial cells, erythrocytes and capillary cells. In the gastric mucosa parietal cells, surface mucous cells and neck mucous cells were highly active. In the small intestine only a few epithelial cells on the villi and in the upper part of the crypts showed enzyme activity. They seemed to be randomly scattered among inactive ones. It is not clear at present if they represent a distinct cell type or specialized absorptive cells. In the proximal colon most surface epithelial cells were highly active (goblet cells were inactive), whereas the surface cells in the distal colon showed less activity with a more varying degree of staining. In the cecum enzyme activity was found in the surface epithelium and in the upper part of the crypts, the staining being most marked at the luminal border of the surface cells. The staining reaction was completely inhibited in all tissues by 10 μM acetazolamide, except for the luminal staining of the cecum, which was inhibited only by 100 μM acetazolamide. This indicates the presence of high concentrations of carbonic anhydrase, probably of the "low activity" form, at this locus. Mucosal scrapings were taken from the intestinal tissues, homogenized and assayed for carbonic anhydrase activity by a changing-pH indicator method. The results confirm those of previous studies and correlate well with the histochemical findings.

Key words: Carbonic anhydrase, histochemistry, stomach, intestine

The presence of carbonic anhydrase in the gastric mucosa is well known, and its distribution and function in this tissue have been extensively studied (see Maren 1967). However much less is known about the enzyme in the other parts of the intestinal tract. Carter and Parsons (1971) found that homogenates of the stomach, proximal colon and cecum of the guinea-pig all possess high carbonic anhydrase activity whereas the activity of the small intestine and distal colon is low. Similar findings have been reported for the dog and rat (Kamaki and Magee 1964, Maren 1967). The physiological significance of these findings is poorly understood, however (see Carter 1972).

The aim of the present study was to clarify the detailed distribution of the enzyme in the intestinal tract of the guinea-pig, since such data are necessary for a further discussion of the role of the enzyme. For this purpose Hansson's (1967, 1968) cobalt–phosphate method for histochemical demonstration of carbonic anhydrase activity has been used. The specificity of this method has been described in another paper (Lönnerholm 1974). Except for

may also have been of importance that the tissue extracts to be compared were incubated simultaneously and that the assay was performed on the same rectus muscle

The synergistic action of the two branches of the autonomic nervous system in the hypertrophy of the submaxillary glands in rats, the incisors of which were cut was pointed out by Wells and Peronace (1964). The marked gain in weight of the parasympathetically decentralized submaxillary glands shows the role of the sympathetic nerve the enlargement of the sublingual glands was probably entirely dependent on the parasympathetic nerve, since the secretory cells of this gland are considered to lack an adrenergic nerve supply (Norberg and Olson 1965 Ohlin and Perec 1965)

This work was supported by grants from the Faculty of Medicine in Lund

References

EKSTRÖM, J *Choline acetyltransferase in salivary glands and some other organs and its dependence on nervous activity* Lund. Rahms boktryckeri. 1975 1–49 (Thesis.)

EKSTRÖM, J Choline acetyltransferase activity and weight of salivary glands in rats at different ages *Acta physiol scand.* 1976. *98* 185–191

EKSTRÖM, J and J HOLMBERG, Effect of decentralization on the choline acetyltransferase of the canine parotid gland *J Physiol* (Lond.) 1972. *222.* 93–94P

MOLINOFF P B and J AXELROD, Biochemistry of catecholamines. *Ann Rev Biochem.* 1971 *40* 465–500

NORBERG K. A. and L. OLSON, Adrenergic innervation of the salivary glands in the rat. *Z Zellforsch* 1965 *68* 183–189

NORDENFELT I Choline acetylase in normal and denervated salivary glands *Quart J exp Physiol* 1963 *48* 67 79

NORDENFELT I Effect of preganglionic parasympathetic denervation on choline acetylase in postganglionic neurones. *Acta Univ Lund.* II 1964. No 16. 1 7

NORDENFELT I., *Acetylcholine synthesis in salivary glands.* Lund Håkan Ohlssons boktryckeri. 1965 1 25. (Thesis.)

OHLIN P and C. J PEREC, Salivary secretion of the major sublingual gland of rats. *Experientia* (Basel) 1965. *21* 408.

OHLIN, P and C. J PEREC, Choline acetylase in normal and denervated submaxillary glands of rats after repeated teeth amputations. *Acta physiol. scand* 1967 *69* 134–139

THOENEN, H Trans-synaptic enzyme induction. *Life Sci.* 1974 *14* 223–235

WELLS, H. and A. A. V PERONACE, Synergistic autonomic nervous regulation of accelerated salivary gland growth in rats. *Amer J Physiol* 1964 *207* 313–318.

ZIEHER, L. M and A. PELLEGRINO DE IRALDI Central control of noradrenaline content in rat pineal and submaxillary glands. *Life Sci.* 1966. *5* 155–161

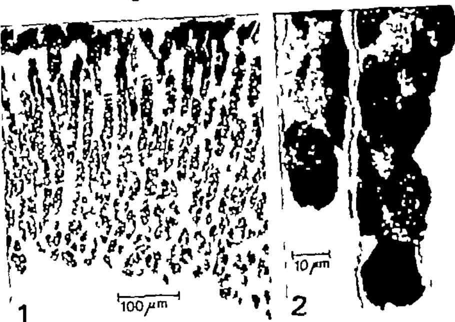


Fig. 1 Gastric mucosa, corpus. Note heavy and uniform staining of the surface epithelium (at the top of the picture). The cells of the glands show varying degree of staining. Fixed, 8 μm thick section. Incubation time 6 min.

Fig. 2 Gastric glands. Stained parietal cells are seen among unstained or weakly stained chief cells. Fixed, 4 μm thick section on Millipore filter. Incubation time 9 min.

Stomach. The carbonic anhydrase activity of gastric mucosal homogenates was the highest found in any intestinal tissues (Table I). Histochemically heavy staining was seen in the surface mucous cells, including those lining the gastric pits (Fig. 1). The whole cells were stained, sometimes with an accumulation of the deposits at the cell borders. The neck mucous cells were similarly stained. In the lower part of the gastric glands, the parietal cells

TABLE I Carbonic anhydrase activity of mucosal homogenates.

Tissue	Enzyme units/g wet weight
whole blood[a]	1 280 _ 639
gastric corpus	2 280 ± 49
jejunum	63 ± 26
ileum	57 14
caecum	548 ± 216
proximal colon	1 215 ± 433
distal colon	96 ± 21

Values are means ± S.E. for 4 animals.
[a] Enzyme units/ml whole blood.

The nomenclature of Itō (1967) has been used.

the stomach, previous histochemical studies are scarce (Korhonen *et al* 1966, Cassidy and Lightfoot 1974) The enzyme activity of mucosal homogenates of the intestinal tissues was also determined, using a changing pH indicator method.

Methods

Tissue preparation. Nonfasted, female guinea-pigs, weighing 300–600 g, were used. They were fed pellets (Ewos avelsfoder kanin och marsvin, Astra Ewos AB Södertälje, Sweden) and water to which ascorbic acid had been added *ad libitum* They were killed by a blow on the head. The abdomen and the thorax were rapidly opened and a cannula was introduced i to the ascendent aorta. Perfusion with cold 0.9% NaCl started after the portal vein had been opened to secure free flow and lasted until the abdominal vessels were macroscopically blood-free.

The stomach and cecum were removed, opened, and the contents washed away by agitating the tissue in a large volume of 0.9% NaCl. Samples were taken from the gastric corpus and from the middle part of the cecum. I testinal segments were used with o without previous perfusion of the intestinal lumen with 0.9% NaCl Jejunal samples were taken 5 to 20 cm distal to the duodenojejunal flexure and ileal samples 5 to 20 cm proximal to the ileocolic valve. Proximal colonic samples were taken 5 to 10 cm distal to the ileocolic valve and distal colonic samples 5 to 10 cm proximal to the a us.

Histochemical staining procedure

Fixed and unfixed tissues from 10 animals were used. Unfixed tissues were immediately frozen in isopentane cooled with a mixture of ethanol and solid CO_2. They were stored in small plastic bags at −70 C fo days or weeks before use

Fixed tissues were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7 4) at 4°C for 4-5 hrs. The glutaraldehyde was prepared from 50% stock solution by a one stage vacuum dist llation, and the purity of the distillate was checked by ultraviolet spectrophotometry (Anderson 1967). The tissues were then briefly rinsed in 0.2 M sucrose + 0.05 M phosphat buffer (pH 7 4) and frozen as described above

Sections were cut in a cryostat at −20°C. They were stained for carbonic a hydrase activity according to the cobalt–phosphate method of Hansson (1967 1968) with only minor mod fications. In this method sections are floated on med um containing N HCO_3, $CoSO_4$, KH_2PO and H_2SO A compound containing cobalt and phosphate is deposited at carbonic anhydrase sites and is converted t CoS. Thus, a black precipitate is formed where enzyme is present. The procedure is described in a paper by Lönnerholm (1974) with discussion of the specificity of the method

Sections with and without counterstaining with hemato ylin and eosin were prepared.

Throughout the work, the specificity of the staining procedure was checked by incubation of sections in the presence of 10 μM acetazolamide (Diamox® American Cyanamid Company Pearl River N Y USA) a specific inhibitor of carbonic anhydrase (Maren 1967).

Biochemical assay of carbonic anhydrase activity

Blood samples were taken by cardiac puncture nd collected int a heparinized test tube The erythrocytes were hemolysed by addi g a large amount of distilled water

Tissues were prepared as described above, and then gently blotted on absorbent paper to remove excess fluid. Mucosal scrapings were taken with a knife blade. They were weighed and homogenized in 9 parts of distilled water containing 1 mM EDTA (sodium salt) to protect the enzyme from inactivation by hea y metal ions. A tight fitting Teflon plunger in a glass tube was used.

The activity of the hemolysates and the whole homogenates was determined by the cha gi g pH method of Philpot and Philpot (1936) as described in detail by Maren *et al* (1954). The activity of sample as calculated from 1–2 determinations on each of 3–4 dilution of the sample. The assays were perf rmed t 2°C.

Results and Discussion

For the histochemical work fixed and unfixed tissues were used as described above. The cellular structure was much better preserved in the fixed sections, allowing more detailed

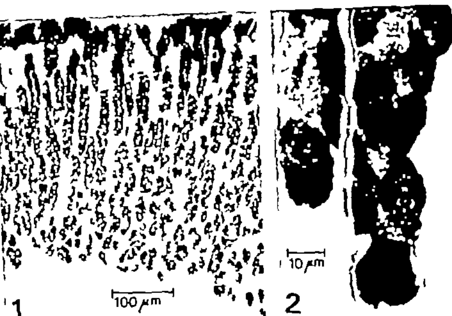


Fig. 1 Gastric mucosa, corpus. Note heavy and uniform staining of the surface epithelium (at the top of the picture). The cells of the glands show varying degree of staining. Fixed, 8 μm thick section. Incubation time 6 min.

Fig. 2. Gastric glands. Stained parietal cells are seen among unstained or weakly stained chief cells. Fixed, 4 μm thick section on Millipore filter. Incubation time 9 min.

Stomach. The carbonic anhydrase activity of gastric mucosal homogenates was the highest found in any intestinal tissues (Table I). Histochemically heavy staining was seen in the surface mucous cells, including those lining the gastric pits (Fig. 1). The whole cells were stained, sometimes with an accumulation of the deposits at the cell borders. The neck mucous cells were similarly stained. In the lower part of the gastric glands, the parietal cells

TABLE I. Carbonic anhydrase activity of mucosal homogenates.

Tissue	Enzyme units/g wet weight
Whole blood[1]	1 290 ± 639
gastric corpus	2 280 ± 492
jejunum	63 ± 26
ileum	57 ± 14
caecum	548 ± 216
proximal colon	1 215 ± 433
distal colon	96 + 21

Values are means ± S.E. for 4 animals.
[1] Enzyme units/ml whole blood.

The nomenclature of Ito (1967) has been used.

were the most active ones (Fig. 2) Their cytoplasm showed a net-like staining pattern, whi probably corresponds to the so-called intracellular canaliculi (see Ito 1967) The chief ce appeared unstained or sometimes weakly stained along the cell borders.

The presence of large amounts of carbonic anhydrase in the mammalian gastric muco was an early discovery (Davenport and Fisher 1938) Carter and Parsons (1971) measur the concentration of the two major isoenzymes of carbonic anhydrase in the intestinal tra of the guinea pig, and found that the gastric mucosa contains much "high activity isoe zyme, but only a negligible amount of "low activity" isoenzyme. The enzyme is suppos to have a role in gastric acid secretion since acetazolamide, a specific inhibitor of the e zyme, reduces the output of hydrogen ions (Janowitz *et al* 1952).

High enzyme activity in the parietal cells has previously been demonstrated in the guine pig and other mammals by histochemical (Vollrath 1959 Hansson 1968 Cross 1970, Wi born *et al* 1974) and other techniques (Davenport 1939 Boass and Wilson 1964). The pre ent data confirm these findings and give further support for the idea that the parietal cel secrete hydrogen ions.

The present demonstration of considerable carbonic anhydrase activity in the cells of th surface epithelium and the neck mucous cells is in agreement with the findings of Vollrat (1959) in the guinea-pig and Boass and Wilson (1964) in the rat Others have reported n or low activity in these cells (Davenport 1939 Korhonen *et al* 1966, Petermann *et a* 1972) The role for the enzyme in the surface and neck mucous cells is not clear at presen It has recently been shown however that the frog fundic mucosa is capable of secreting a alkaline fluid (Flemström 1976) This was demonstrated after the acid secretion had bee blocked by inhibitors not affecting carbonic anhydrase. When acetazolamide was adde the alkaline secretion was inhibited These findings, together with the present ones, sugge that carbonic anhydrase in the surface epithelium has a role in alkaline secretion, which ma protect these cells from damage due to a low gastric intraluminal pH

Small intestine Mucosal homogenates of the jejunum and ileum showed low enzym activity which was only about 1/40 of that of the stomach (Table I) The histochemical find ings in the jejunum and ileum were similar and are described together

The epithelium of the villi and the crypts showed no activity except for a small numbe of cells which were stained distinctly (Fig. 3 4) These slender and elongated cells were ofte found to reach from the intestinal lumen to the epithelial basement membrane. They seeme to be fairly evenly distributed on the villi but were less common in the crypts. In the bottom of the crypts no stained cells were found No attempt was made to quantify the number o stained cells, but a rough estimate is that 1/10 to 1/30 of the cells of the villus epitheliun were stained. Obvious differences between the number of stained cells in the different parts of the small intestine were not observed.

The whole cells were stained in fixed sections (Fig. 4) whereas the staining was restricted to the luminal part of the cells in unfixed sections (Fig. 5) This might be due to loss of en zyme from the cytoplasm of unfixed cells.

Carter and Parsons (1971) found small amounts of high activity" and "low activity carbonic anhydrase in the small-intestinal mucosa. The present histochemical findings show that the enzyme is localized to a certain population of mucosal cells, scattered in the villus

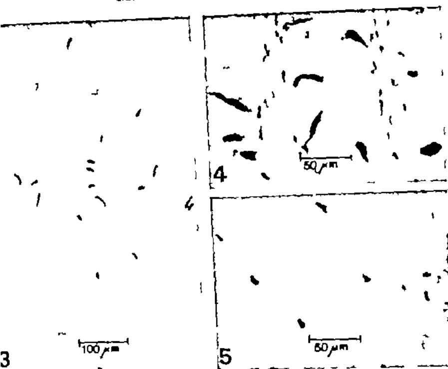


Fig. 3. Mucosa of the distal ileum. A few epithelial cells on the villi and in the upper part of the crypts are stained. Fixed, 8 μm thick section. Incubation time 4 min. Counterstaining with hematoxylin and eosin.

Fig. 4. Lower part of branching villus in the proximal jejunum. A few epithelial cells are stained. When sectioned longitudinally they can be seen to reach from the epithelial basement membrane to the intestinal lumen. There is also some capillary staining. Fixed, 8 μm thick section. Incubation time 6 min. Counterstaining with hematoxylin and eosin.

Fig. 5. Closely packed villi in distal ileal mucosa. A few epithelial cells are stained at the luminal cell border. Unfixed, 4 μm thick section on Millipore® filter. Incubation time 3 min. Counterstaining with hematoxylin and eosin.

epithelium. The low activity of the mucosal homogenates corresponds to the small number of active cells.

Earlier histochemical studies are confined to the small intestine of the rat (Korhonen et al. 1966, Cassidy et al. 1972, Cassidy and Lightfoot 1974). The results differ from the present ones since these authors reported a uniform staining of the villus epithelium, at least in the jejunum. This discrepancy might possibly be due to the use of different species or techniques.

The identity of the carbonic anhydrase containing cells found here has not been clarified yet. They are obviously not identical with goblet cells, however. They might represent specialized absorptive cells, or a distinct cell type, for example some kind of endocrine cell. Further attempts to characterize the carbonic anhydrase cells by various staining techniques and studies at the ultrastructural level are in progress. The discussion of the role of the enzyme in the small intestinal mucosa must await the result of such studies.

Cecum. The carbonic anhydrase activity of the cecal mucosa was about 1/4 of that of the

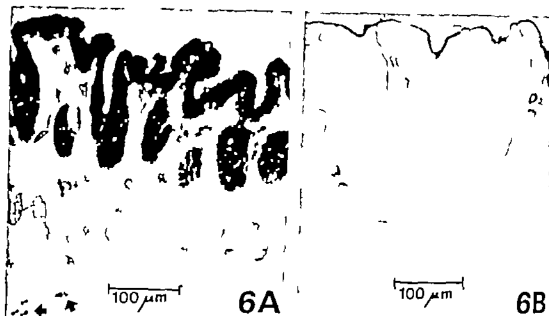


Fig. 6 Cecal mucosa. *6 A* The cells of the surface epithelium and the upper part of the crypts are intensely stained. Arrows indicate stained erythrocytes. *6 B* Addition of 10 μM acetazolamide to the incubation medium inhibits all staining except at the luminal border of the surface cells. Fixed, 8 μm thick sections. Incubation time 6 min. Weak counterstaining with hematoxylin and eosin.

gastric mucosa (Table I) Histochemically intense staining was found in the cells of the surface epithelium and the upper part of the crypts, whereas those lining the lower part of the crypts were unstained (Fig. 6 A) With short incubation times the luminal part of the cells showed the most intense staining. With longer times this was obscured by a heavy staining of the whole cells.

The addition of 10 μM acetazolamide to the incubation medium blocked the cytoplasmic staining, but not the staining at the luminal cell border of the surface cells (Fig. 6 B). It took 100 μM acetazolamide to inhibit this staining. This is remarkable since the low concentration of 10 μM of the inhibitor completely abolished the staining in the other intestinal tissues. To exclude the possibility that the luminal staining was non-enzymic and inhibited non-specifically by the high concentration of the inhibitor sections were also incubated without any substrate (*i.e.* bicarbonate) in the medium. This produced completely unstained sections.

Previous histochemical studies of the cecum seem to be lacking. However Carter and Parsons (1971) have reported that the cecal mucosa contains predominantly a "low activity" isoenzyme of carbonic anhydrase, its concentration being higher than in any other intestinal tissue of the guinea pig. This low activity" isoenzyme is many times less sensitive to acetazolamide than the "high activity" isoenzyme (Carter and Parsons 1972).

That the cecal mucosa indeed possesses considerable enzyme activity is shown here biochemically and histochemically The present finding that the staining at the luminal border of the surface cells was abolished only by high concentrations of acetazolamide would indicate that high concentrations of carbonic anhydrase, probably of the "low activity" ... This localization of the enzyme strongly suggests a role in transport

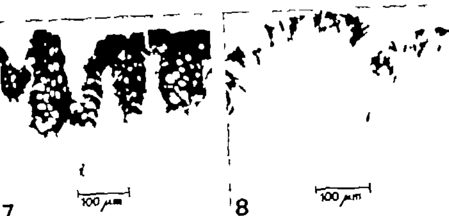


Fig. 7. Proximal colonic mucosa. Intense staining in the surface epithelium and the upper part of the crypts. Unstained goblet cells are seen among the stained cells. Fixed 8 µm thick section. Incubation time 6 min. Counterstaining with haematoxylin and eosin.
Fig. 8. Distal colonic mucosa. The surface epithelial cells show varying degree of staining. Fixed, 8 µm thick section. Incubation time 6 min. Counterstaining with haematoxylin and eosin.

processes between the intestinal lumen and the cells. It has been proposed that the "low activity" carbonic anhydrase of the cecum is concerned with the absorption of products of microbial fermentation, such as ammonia or volatile fatty acids (Carter and Parsons 1971), but this remains hypothetical.

Colon. Carbonic anhydrase activity almost comparable to that of the stomach was found in the proximal colon (Table I). The distal colon showed a much lower enzyme activity. This is in agreement with previous findings in the guinea-pig (Carter and Parsons 1971) and in the dog and rat (Kuriaki and Magee 1964).

In the proximal colon heavy staining was found in most cells of the surface epithelium and the upper part of the crypts (Fig. 7). Some unstained, barrel-shaped cells were seen among the stained ones, probably representing goblet cells. The cells in the lower part of the crypts were completely unstained.

In the distal colon the staining of the surface epithelial cells varied markedly. Distinctly stained cells appeared among unstained ones, and intermediate forms showing weak staining were also seen (Fig. 8). The crypt cells were unstained.

These findings agree reasonably well with previous histochemical studies in the rat colon reported by Korhonen *et al.* (1966) and Helander (1975).

The colonic mucosa is capable of absorbing sodium and chloride against large concentration gradients, with a concomitant secretion of bicarbonate. Acetazolamide has been reported to reduce the secretion of bicarbonate and the absorption of sodium, chloride and water in the rat colon (Parsons 1956, Phillips and Schmalz 1970). These findings suggest that carbonic anhydrase may have a role in the exchange of bicarbonate and chloride across the mucosa, and that this anion exchange may be related to the mechanism of sodium and water transport in the colon. The present finding that carbonic anhydrase is localized to the surface cells of the colonic mucosa is in line with such a role for the enzyme.

It is presently not understood, however, why large amounts of the "high activity" and

"low activity" isoenzyme are present in the proximal colonic mucosa, while the distal colonic mucosa possesses only or almost only "low activity" carbonic anhydrase (Carter and Parsons 1971). The role for the "low activity" isoenzyme could possibly be the same as that discussed for the cecal enzyme, i.e. to participate in the absorption of products of microbial fermentation (see above).

Non-epithelial cells. Except for epithelial cells, erythrocytes and capillaries were the only stained structures. The few erythrocytes left in the tissues after the perfusion with saline (see Methods) were heavily stained (Fig. 6 A). The capillary walls showed a varying degree of staining. This might possibly be due to the presence of a varying number of erythrocytes inside the vessels.

I am grateful to Mrs. Mona Schenholm for skilful technical assistance.
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References

ANDERSON, P. J. Purification and quantitation of glutaraldehyde and its effects on several enzyme activities in skeletal muscle. *J. Histochem. Cytochem.* 1967. *15.* 652–661.

BOASS, A. and T. H. WILSON, Cellular localization of gastric intrinsic factor in the rat. *Amer. J. Physiol.* 1964. *206.* 783–786.

CARTER, M. J. and D. S. PARSONS, The isoenzymes of carbonic anhydrase: tissue, subcellular distribution and functional significance with particular reference to the intestinal tract. *J. Physiol.* (Lond.) 1971. *215.* 71–94.

CARTER, M. J. Carbonic anhydrase: isoenzymes, properties, distribution, and functional significance. *Biol. Rev.* 1972. *47.* 465–513.

CARTER, M. J. and D. S. PARSONS, The isoenzymes of carbonic anhydrase: kinetic properties with particular reference to the functions in the intestinal tract. *J. Physiol.* (Lond.) 1972. *220.* 465–478.

CASSIDY, M. M., M. J. JACKSON, F. LIGHTFOOT and S. BANK, Carbonic anhydrase and transport mechanisms in rat small intestine. *Fed. Proc.* 1972. *31.* .59.

CASSIDY, M. M. and F. G. LIGHTFOOT, Carbonic anhydrase. In *Electron microscopy of enzymes,* vol. edited by M. A. HAYAT. Van Nostrand Reinhold Company. 1974. 77–108.

CROSS, S. A. M. Ultrastructural localization of carbonic anhydrase in rat stomach parietal cells. *Histochemie* 1970. *22.* 219–.25.

DAVENPORT, H. W. and R. B. FISHER, Carbonic anhydrase in the gastrointestinal mucosa. *J. Physiol.* (Lond.) 1938. *94.* 16 P.

DAVENPORT, H. W. Gastric carbonic anhydrase. *J. Physiol.* (Lond.) 1939. *97.* 32–43.

FLEMSTRÖM, G. Properties of isolated gastric antral and non-acid secreting fundic mucosa. In *Gastric hydrogen ion secretion,* edited by D. K. Kasbekar, G. Sachs and W. S. Rehm. New York. Marcel Dekker Inc. In press.

HANSSON, H. P. J. Histochemical demonstration of carbonic anhydrase activity. *Histochemie* 1967. 112–128.

HANSSON, H. P. J. Histochemical demonstration of carbonic anhydrase activity in some epithelia noted for active transport. *Acta physiol. scand.* 1968. *73.* 427–434.

HELANDER, H. F. Enzyme patterns and protein absorption in rat colon during development. *Acta anat.* (Basel) 1975. *91.* 330–349.

ITO, S., Anatomical structure of the gastric mucosa. In *Handbook of Physiology,* section 6, vol. II. Secretion, editor C. F. Code. Washington D.C., American Physiological Society. 1967. 705–741.

JANOWITZ, H. D., J. COLCHER and F. HOLLANDER, Inhibition of gastric secretion in dogs by carbonic anhydrase inhibitor 2-acetylamino-1,3,4-thiadiazol-5-sulfonamide. *Amer. J. Physiol.* 1952. *171.* 325–330.

KORHONEN, L. K., E. KORHONEN and M. HYYPPÄ, Histochemical demonstration of carbonic anhydrase activity in the alimentary canal. *Histochemie* 1966. *6.* 168–172.

KURIAKI, K. and D. F. MAGEE, On the carbonic anhydrase activity of the alimentary canal and pancreas. *Life Sci.* 1964. *3.* 1377–138..

…ONNEHOLM, G., Carbonic anhydrase histochemistry critical study of Hansson cobalt–phosphate method. *Acta physiol. scand.* 1974, Suppl. 418, 1–43.

…LAUN, T. H., V. L. Ams and E. M. Bailey. Carbonic anhydrase inhibition. II. A method for determination of carbonic anhydrase inhibitors, particularly of Diamox. *Bull. Johns Hopk. Hosp.* 1954, 95, 244–…53.

…ers, T. H. Carbonic anhydrase: Chemistry, physiology and inhibition. *Physiol. Rev.* 1967, 47, 595–…11.

…ther, T. F. A critical evaluation of the histochemical methods for carbonic anhydrase. *J. Histochem. Cytochem.* 1972, 20, 319–330.

…dona, D. S. The absorption of bicarbonate-saline solutions by the small intestine and colon of the white rat. *Quart. J. exp. Physiol.* 1956, 41, 410–420.

…rma…n, J., D. Lorenz and A. Dorn, Histochemische Untersuchungen der Magenschleimhaut von Ratten vor und nach trunkulärer Vagotomie. *Acta histochem.* (Jena) 1972, 43, 71–82.

…umps, S. F. and P. F. Schwaltz, Bicarbonate secretion by rat colon, effect of intraluminal chloride and acetazolamide. *Proc. Soc. exp. Biol.* (N.Y.) 1970, 135, 116–122.

…lpot, F. J. and J. St. L. Philpot. A modified colorimetric estimation of carbonic anhydrase. *Biochem. J.* …936, 30, 2191–2194.

…llrath, L. Über Entwicklung und Funktion der Belegzellen der Magendrüsen. *Z. Zellforsch.* 1959 …0, 36–60.

…ndon, W. B., L. L. Sello, J. and C. M. Girard, Variation in the pattern of carbonic anhydrase activity in the cells of the gastric glands. *Histochemistry* 1974, 39, 289–300.

Acta physiol. scand. 1977 99 62–74
From the Department of Zoophysiology University of Göteborg, Sweden

Regulation of the Heart of a Teleost, *Gadus morhua*, by Autonomic Nerves and Circulating Catecholamines

By

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Abstract

HOLMGREN S *Regulation of the heart of a teleost Gadus morhua, by autonomic nerves circulating catecholamines* Acta physiol scand 1977 99 62–74

The cod heart regulation has been investigated by anatomical studies, fluorescent histochemistry stimulation and drug effects on the perfused heart and electrically paced strips. The effect on the he catecholamine release from the head kidney has been studied in a perfused head kidney and heart paration. Branches of the vagi and the fused 1st and 2nd spinal nerves innervate the heart. Specif fluorescent fibres were found in these nerve branches, in the ducts of Cuvier all parts of the heart, b arteriosus and the ventral aorta. Weakly tofluorescent ganglion cells surrounded by specifically flo cent varicose fibres were present in the walls of the sinus venosus. Vagal stimulation caused bradyc altered after atropine to tachycardia. Spinal nerve and sympathetic chain stimulation also induced t cardia, as did provoked catecholamine release from the head kidney The tachycardia could be blocke propranolol. Atropine increased and propran lol decreased basic heart rate nd contraction f Adrenergic drugs accelerated and acetylcholine retarded the perfused heart. A positive inotropic e was obtained with adrenergic drugs on isolated heart strips, while acetylcholine produced a negativ otropic effect on atrial but not ventricular strips. It is concluded from thi study that the cod heart an inhibitory cholinergic nerve supply *via* the vagi and an excitatory adrenergic supply *via* both the and the first spinal nerves. Sympathetically controlled release of catecholamines from the chrom tissue in the head kidney may also play a important role in the heart regulatio

Key ords: T leost heart, autonomic innervation, catecholamines

Numerous authors have throughout the years demonstrated the presence of a vagal hibitory cholinergic innervation of the teleost heart (for references see Von Skramlik 19 Laurent 1962, Campbell 1970) The cause of excitation of the heart is less clear and see to vary with species. The possibility of increasing the heart rate by lowering the choliner vagal tonus has been frequently discussed (McWilliam 1885 Von Skramlik 1935 Kulb 1957 Rodinov 1959 Laurent 1962, Randall 1966). Presence of adrenergic receptors (Brinl 1933 Östlund 1954 Falck *et al* 1966 Gannon and Burnstock 1969 Gannon 1971 Col and Santer 1973) indicates ability of regulation *via* circulating catecholamines and/or a renergic nerves. No adrenergic nerves are found in the heart of the plaice, *Pleuronect platessa* (Santer 1972), and the excitatory effects of vagal stimulation at low frequencies a

lieved to be rebound effects (Cobb and Santer 1973). The trout (*Salmo trutta*, *S. irideus*) heart is, however innervated by sympathetic adrenergic fibres running in the vagus nerve; these are presumed to cause the excitatory effect on vagal stimulation with low frequencies after atropine (Yamauchi and Burnstock 1968, Gannon and Burnstock 1969 Gannon 1971).

In this study the cardiac innervation and the possibility of cardiac regulation by circulating catecholamines in the cod, *Gadus morhua*, have been investigated.

Materials and methods

Cod, *Gadus morhua*, body weight 200–2 000 g, kept in aerated, circulating sea water at 10°C were used for this study. Fish, which were used for perfusion expts., were before killing injected with heparin 5000 U pro kg b.wt. in the caudal vessels. The fish were killed by a sharp blow on the head. A cod Ringer solution (Holmgren and Nilsson 1974) was used as perfusion fluid and saline for isolated strips.

Anatomical studies. For dissection fish weighing 1–2 kg were used. They were carefully opened by an incision behind and parallel to the 4th gill arch for studies of the innervation of the heart. 1% osmic acid was used for staining of the nerve fibres (Young 1931).

Fluorescence histochemistry. Tissue pieces were rapidly dissected out from freshly killed fish and quick-frozen in liquid propane cooled in liquid nitrogen. They were freeze-dried and prepared according to the method described by Falck and Owman (1965). Since adrenaline is the dominant catecholamine in the heart (Euler and Fänge 1961, Abrahamsson and Nilsson 1976), 3 h incubation in formaldehyde vapour at 80°C was used. The pieces were embedded in paraffin wax, sections of 10 μm were cut, mounted in Entellan (Merck) and viewed in a Leitz Orthoplan microscope with top light illumination and a barrier filter at 460 nm. With this treatment the presence of catecholamines in the preparations could be identified from the yellow-green ("specific") fluorescence. Photographs were taken with a Leitz-Orthomat automatic camera on Kodak Tri-X film.

Perfused head kidney and heart preparation. The fish was opened ventrally, the hepatic veins, the left subintestinal vein and the right duct of Cuvier ligated and the swim-bladder opened. The inflow polythene catheter (PE 60) was inserted into the left posterior cardinal vein along the length of the swim-bladder up to the head kidney. The perfusion fluid was then allowed to pass through the head kidney and via the duct of Cuvier through the heart. An outflow catheter was inserted into the ventral aorta. The perfusion was performed with constant flow rate, achieved with a BRAUN Perfusor with cod Ringer solution. The flow rate used was 0.4 ml/min or 1.0 ml/min depending on animal size. Pressure variations in the outflow of saline were measured by a Statham P 23 transducer. The pulse record was fed into a Grass 7P4 Tachograph, which displayed the heart rate as a beat-to-beat record. A Grass Polygraph Recorder model 79B was used for the recordings. The coeliac ganglion, where fibres from the left sympathetic chain pass through to the chromaffin tissue in the walls of the head kidney parts of the posterior cardinal vein (Nilsson 1976), was carefully located and placed on platinum electrodes for stimulation.

Heart perfusion with constant pressure. The fish was placed ventral side up and opened ventrally and behind the 4th gill arch on each side. The hepatic veins were ligated. Polyethene catheters (PE 60) were inserted into the left duct of Cuvier and the ventral aorta for inflow and outflow respectively of perfusion fluid. Perfusion with cod Ringer solution was performed with constant pressure as described by Nilsson and Grove (1974). The inflow pressure was kept between 5 and 15 cm of water. Within this range the heart rate was found to be independent of the perfusion pressure. Johansen (1962) also reports that the cod heart reacts to increased venous return with increased beat volume and keeps the heart rate constant. The outflow pressure was kept above 20 cm of water.

When effects of nerve stimulation were tested the pericard was left intact, and the heart rate was obtained as described above for the perfused head kidney and heart preparation. Platinum electrodes for vagal stimulation were placed either around the whole vagus nerve ca 10 mm from the skull or around the cardiac branch at the duct of Cuvier ca 3 mm from the sinus venosus. Electrodes for stimulation of the branch of the fused 1st and 2nd spinal nerves running parallel to the duct of Cuvier were placed between 20 and 30 mm from the outflow of the skull. For stimulation of the right sympathetic chain the right side of the body wall of the fish was cut away. The parts of the sympathetic chain near the coeliac ganglion were dissected free from surrounding head kidney tissues and stimulated with platinum electrodes.

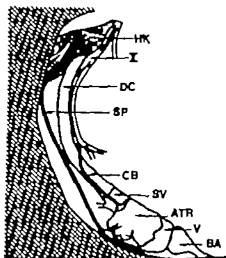


Fig. 1 Lateral view of the right side of the cardiac sp of the cod *Gadus morhua*. The heart is innervated by cardiac branch of the vagus nerve. Fibres ha e also b seen to leave the branch of the fused 1st and 2nd sp nerves running down along the duct of Cuvier and forw ventral of the pericard, and to enter the duct of Cuvie the atrial tissue directly *via* the pericard. Legend: ATR atrium, BA = bulbus arteriosus, CB = cardiac branch of t vagus nerve, DC = duct of Cuvier SP = branch of the fu 1st and 2nd spinal nerves, SV = sinus enosus, HK k kidney V = ventricle, X = vagus nerve.

In experiments where drug effects on the heart rate have been tested, the right duct of Cuvier was lig and the pericard opened. A piece of cotton was placed around the ventricle and tied to a Grass FT trandsducer connected to a Grass Polygraph Recorder model 79B for recording of the heart movemen The heart rate was displayed as a beat-to-beat record by a Grass 7P4 Tachograph.

Agonists were added in single doses to the lower funnel of the perfusion apparatus (Nilsson and Gr 1974) to prevent the drugs from dilution in the major bulk of the perfusion fluid. Antagonists were add to the entire perfusion fluid 15 min before the followi g nerve stimulation or addition of agonists. Do response experiments were made with the agonists and their effects are presented as pD_2-values (v Rossum 1963). Blocking effects of antagonists are, when possible calculated as pA_2- and pA_{10}-val (Schild 1947) as described by Holmgren and Nilsson (1975).

Isolated heart strip experiments Longitudinal strips, ca mm wide and 5–10 mm long were cut fro both trium and ventricle. They were mounted in organ baths in cod Ringer solution at 10°C, bubb with a mixture of 3% CO_2 nd 97% O_2, and connected to Grass FT 03 transducers for recording of tensi on a Grass Polygraph Recorder model 7 B. Tension was measured in millipond (mp = 9.81×10^{-4} The strips were electrically paced by Grass SD 9 Stimulators *via* silver electrodes. Cumulati dose-respon curves were made, and effects of drugs were calculated as described for the perfused heart.

Drugs. The drugs used were Acetylcholine chloride, 1-adrenaline bitartrate, atropine sulphate, isoprenaline hydrochloride, 1-noradrenaline bitartrate, propranolol hydrochloride.

Statistics. Means are given ± standard erro (S.E.), numbers of expts. are indicated as n. Not more th one experiment is done on one fish, except for the isolated strips, where two strips were taken from ea heart chamber Significance of differences in potency between drugs has been calculated by Student t-test.

Results

Dissection with osmic acid

The cod heart is innervated by a cardiac branch of each vagus nerve. The fibres follow th duct of Cuvier on both sides into the pericard and are spread out on the sinus venosu one part on the dorsal and one part on the ventral side, in a region near the atrium (Fig. 1)

Fluorescence histochemistry

Bundles of specifically fluorescent fibres are found both in the branches of the fused 1s and 2nd spinal nerves running along the ducts of Cuvier (Fig. 2 a) and in the cardiac branche of the vagi (Fig. 2 b).

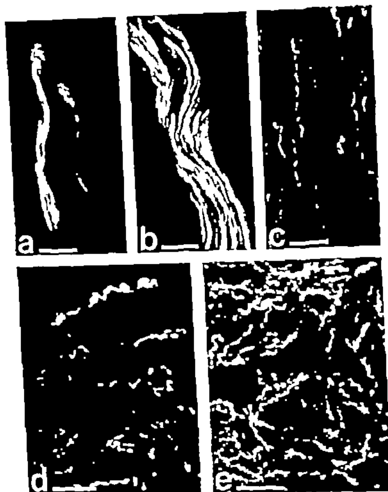


Fig. Microphotographs of fluorescent histochemical preparations. Formaldehyde treatment for 3 h. Longitudinal section of the branch of the fused 1st and 2nd spinal nerves running parallel to the duct of Cuvier shows bundles of specifically fluorescent fibres. b. Longitudinal section of the cardiac branch of the vagus nerv shows bundle of specifically fluorescent fibres running amongst non-fluorescent fibres. c, d and Smooth and varicose fluorescent fibres are abundant in the walls of the ducts of Cuvier (c), the sinus venosus (d) and the bulbus arteriosus (e). Calibration bar in all pictures 50 µm.

The walls of the ducts of Cuvier as well as the walls of sinus venosus are densely innervated by specifically fluorescent, varicose fibres (Fig. 2 c, 2 d). In the part of sinus venosus bordering to the atrium, aggregations of ganglion cells are located. These seem to lack specific fluorescence themselves, but are surrounded by fluorescent nerve terminals (Fig. 3 a).

The tissues of the atrium and the ventricle show a heavy autofluorescence, which made it difficult to find and photograph the weaker specific fluorescence from the catecholamines. However fluorescent fibres are found throughout the atrial tissue, being most frequently found in the outermost layer of the atrium and in the parts nearest sinus venosus (Fig. 3 b). Fluorescent fibres are also seen running between the atrial and the ventricular tissue (Fig.

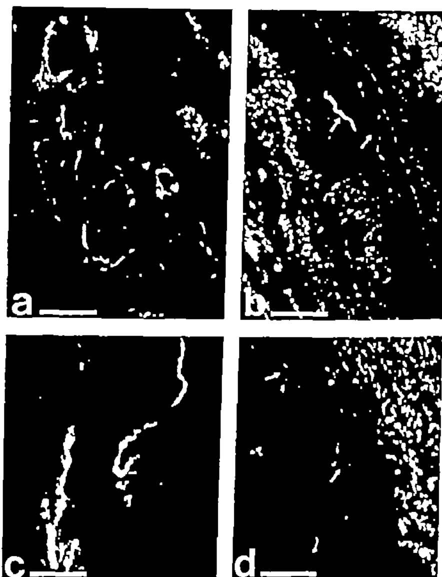


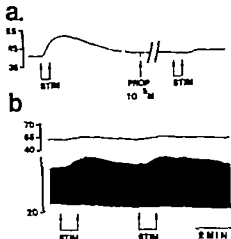
Fig. 3 Microtophographs of fluorescent histochemical preparations of the cod heart. Formaldehyd ment for 3 h. *a*. Aggregation of ganglio cells i the wall of sinus venosus, bordering the trial tissue right corner). The ganglion cells lack specific fluorescence themselves, but are surrounded by spec fluorescent varicose fibres. *b*. The trial tissue show heavy autofluorescence, but specifically fluo fibres can be seen (arrows). *c* Specific fluorescence the tissues surrounding the openi g betwe atrium and the ventricle *d* Varicose specifically fluorescent fibres (arrows) in the outermost part ventricle, where the autofluorescence is bsent.

3 c) The ventricular tissue is much less innervated by fluorescent fibres than the tissue, but specifically fluorescent terminals are found especially in the outermost (Fig. 3 d).

Bulbus arteriosus (Fig. 2 e) and the ventral aorta are densely innervated by specifi fluorescent fibres.

Perfused head kidney and heart preparation

The heart rate during perfusion with constant flow rate through the head kidney and heart was in 15 fish tested 49 ± 3 bpm. In 11 of these fish stimulation of the satellite gang



...g. 4. Effects on two different cod hearts of stimulation of the nerves to the chromaffin tissue in the ...and kidney during perfusion of the head kidney and the heart. Time bar = 2 min. a. Recording of the heart rate. Stimulation (STIM) with 20 Hz, 1 ms and 10 V for 30 s, before and 15 min after addition of propranolol to final concentration of 10^{-4} M ...the perfusion fluid. b. Upper tracing: heart rate, lower tracing: outflow pressure. The 'diastolic' ...flow pressure was adjusted to 20 cm H_2O or ...bove. No measuring of the absolute 'systolic' ...outflow pressure was made due to the inaccuracy ...caused by the experimental setup and since only ...relative variations in contraction force were of ...for this study. Stimulation with 20 Hz, ...ms and 20 V for 60 s. A more pronounced inotropic ...effect is noticed than the chronotropic effect is ...low.

...20 Hz, 1 ms and 10 V for 30 s caused tachycardia (Fig. 4 a), and sometimes an increase ...outflow pressure from the heart (Fig. 4 b). The increase in heart rate after the first stimulation varied from fish to fish between 4 bpm and +25 bpm (mean value +11 ±2 bpm). ...stimulation effect could be repeated several times, but with a slow decline in the increase ...heart rate. Slight responses could in the other 4 fishes be obtained with increased voltage ...stimulation time. The increase in outflow pressure (Fig. 4 b) was more pronounced ...than the effect on the heart rate was low.

Propranolol 10^{-6}–10^{-4} M abolished the effects on the heart rate after stimulation of the ...affine ganglion (n = 4, Fig. 4 a). Propranolol 10^{-4} M decreased the spontaneous heart ...by 9 ±2 bpm (n = 4).

...rve stimulations

...imulation of the vagal branches to the heart with 1 ms and 4–10 V with stimulation ...frequencies from 0.5 Hz and upwards caused bradycardia (Fig. 5 a). A stimulation frequency ...12 ±3 Hz (n = 5) (left vagus) or 8 ±1 Hz (n = 6) (right vagus) arrested the heart beat ...completely for a stimulation period of 30 s. Tachycardia was never obtained at any frequency ...tested on the untreated heart. On cessation of vagal inhibition of the heart a transient increase ...heart rate compared to before the stimulation was often observed (Fig. 5 b).

After atropine (10^{-6} M) vagal stimulation induced a positive chronotropic and/or inotropic effect on the heart in 7 out of 15 fish (Fig. 5 c, 5 d). Propranolol (10^{-6}–10^{-4} M) blocked these effects (... 4).

Stimulation of the spinal nerve with 20 Hz, 1 ms and 10–20 V accelerated the heart in ...0 out of 18 fish (Fig. 6 a). Stimulation with low frequencies gave less effect than 10 or 20 Hz. The increase in heart rate after stimulation was abolished by addition of propranolol (10^{-4} M, n = 4) to the perfusion fluid (Fig. 6 b).

When the spinal nerve and the atropinized vagus were tested in the same animal, either one of them, both or none gave a positive chronotropic effect.

Stimulation of the sympathetic chain behind the coeliac ganglion with varying frequencies and duration never affected the heart (... 9). In 3 fish of 12 tested stimulation at the level

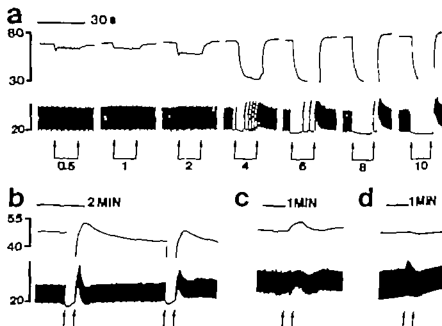


Fig. 5 Effects on the perfused cod heart of stimulation f the vagus nerve. Upper tracings: heart ra Lower tracings recording of outflow pressure Th diastolic' outflow pressure was adjusted to 20 c H O or bove. No measuring of the bsolute systolic outflow pressure was made due to the inaccur caused by the experimental setup and since only the relative variations in contraction f rce ere of inter for this study Arrows indicate stimulation period *a*. Stimulation f the cardiac branch 5 mm from t sinus venosus with 1 ms nd 10 V fo 15 s Numbers indicat the stimulation frequency (Hz) used Brad cardia is obtained at ll frequencies tested *b* Stimulation of the left vagus with 20 Hz, 1 ms and 10 for 30 s. On cessation of vagal stimulatio a transient increase in heart rate above normal as achieve (Can also be noticed in *a*). *d* Stimulation of the left vagus with 10 Hz (*c*) or 20 Hz (*d*), 1 ms and V for 30 s 15 min after addition of atropine to a final concentrati n of 10^{-4} M in the perfusion fluid chronotropic effect was obtained i while in *d*. an inotropic effect can be noticed.

of the coeliac ganglion with 20 Hz, 1 ms and 20 V caused a slight increase in the hea rate (Fig. 6 c)

Chronotropic effects of drugs

Addition of acetylcholine in single doses to the perfusion fluid caused a dose-depende decrease in heart rate (Fig. 7 a) with pD $= 8.27 \pm 0.20$ (n = 13) Atropine blocked this effe (pA $= 9.8 \pm 0.2$, pA $= 8.9 \pm 0.2$, n = 6) Atropine 10^{-4} M also increased the basic hea rate (Fig. 8 b) with up to 15 bpm (mean 5 ± 1 bpm n = 16)

Adrenergic drugs dose-dependently increased the heart rate (Fig. 8 c) with the order o potency isoprenaline (pD $= 10.33 \pm 0.36$ n = 5) > adrenaline (pD $= 10.16 + 0.14$ n = 6) noradrenaline (pD $= 9.22 \pm 0.19$ n = 7 $p < 0.005$ compared to adrenaline) indicating a action via β-adrenergic receptors. The effects of high doses of adrenergic drugs could not b completely washed out, and subsequent dose–response curves showed an irregular decreas in sensitivity No expts. with adrenergic blocking agents have therefore been performed.

Inotropic effects of drugs

Inotropic effects of drugs on the heart were tested on isolated strips, which were paced electrically Stimulation with 1 Hz, 1 ms and 10-20 V for the atria and 0.5 Hz, 1 ms and 10-20 V for the ventricles was found to pace the strips evenly throughout an expt.

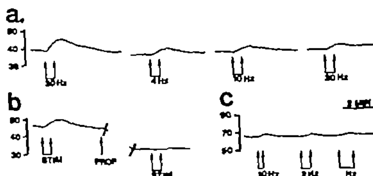


Fig. 6. Recordings of the heart rate of the perfused cod heart. Time bar = min. a. Stimulation of the spinal nerve every 8th minute with varying frequency (Hz), 1 ms duration and 10 V for 30 caused tachycardia. Note the decline in effect of subsequent stimulations by comparing the 1st and the 4th stimulation. b. The effects of stimulation of the spinal nerve with 20 Hz, 1 ms and 10 V for 30 before and 15 min after addition of propranolol to final concentration of 10^{-6} M to the perfusion fluid. The addition of propranolol in this case also produced pronounced decrease in basic heart rate. Stimulation of the sympathetic chain at the level of the coeliac ganglion with 1 ms and 20 V for 30 with 10 Hz, 60 with 2 Hz and 90 with 4 Hz, caused slight increase in heart rate.

Addition of atropine to the bath increased the contraction force of the atrial strips with totally $12 \pm 2\%$ (n 8) and $20 \pm 5\%$ (n 6) after a final atropine concentration in the bath of 10^{-8} M and 10^{-} M respectively (Fig. 8). The ventricular strips were unaffected by atropine up to 10^{-} M (n 8)

Propranolol 10^{-6} M in the bath decreased the contraction force of the atrial strips with 9 3% (n 11) and of the ventricular strips with $4 \pm 1\%$ (n = 11).

Cumulative doses of acetylcholine produced a dose-dependent negative inotropic effect on strips from the atria (pD 5.19 ± 0.17 n 10). $7 \pm 2\%$ (n 10) of the original contraction caused by the electrical stimulation remained after maximal effect of acetylcholine (Fig. 8). Atropine produced a dose-dependent parallel shift to the right of the acetylcholine curve with pA_2 9.0 and pA_{10} 8.1 (n = 7). Acetylcholine 10^{-10}–10^{-4} M had no recordable effects on strips from the ventricle (n 8) (Fig. 8).

Adrenergic drugs produced an increase of the contraction force in both atrial and ventricular strips (Fig. 8), with the order of potency isoprenaline adrenaline > noradrenaline for

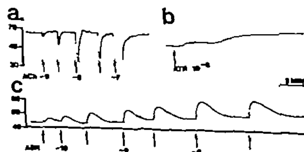


Fig. 7. Chronotropic effects of drugs on the whole cod heart, perfused in situ. Legend: ACh acetylcholine, ADR adrenaline, ATR atropine. Arrows indicate addition of drugs. Agonists are added in subsequent doses of 3 10^{-11} moles, 10^{-10} moles, 3 10^{-10} moles, 10^{-9} moles, etc. Time bar 2 min.

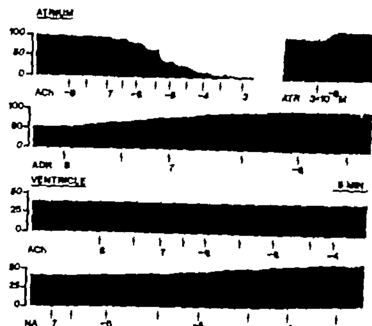


Fig. 8. Inotropic effects of drugs on isolated strips from atria and ventricles, electrically paced at 1 Hz, 1 ms and 10 V and 0.5 Hz, 1 ms and 10 V respectively. Legend: ACh = acetylcholine, ADR = adrenaline, NA = noradrenaline, ATR = atropine. Arrows indicate addition of drugs. Agonists are added in cumulative doses, giving final concentration in the bath of 10^{-8} M, 3×10^{-8} M, 10^{-7} M, 3×10^{-7} M etc. The tension is given in mg. Time bar 5 min.

atrial strips and isoprenaline > adrenaline > noradrenaline for ventricular strips, indicating an effect *via* β-adrenoceptors. pD_2-values are presented in Table I. The effects on the ventricle were, however, irregular from fish to fish: about 50 % of the fish tested gave no recordable response at all.

The effect from the first dose–response curve could seldom be completely washed out, and subsequent dose response curves showed a decrease in positive inotropic effects. Attempts to quantify the effects of blocking agents have therefore not succeeded.

Discussion

The results from this study indicate the presence of a vagal inhibitory cholinergic nerve supply to the cod heart acting on muscarinic receptors in the heart tissue. This is consistent with the situation in other teleosts tested, *e.g.* the trout, *Salmo irideus* (Gannon and Burnstock 1969, Gannon 1971) and the plaice, *Pleuronectes platessa* (Cobb and Santer 1973) (for references see also Laurent 1962, Burnstock 1969). Both chronotropic effects (whole perfused heart) and inotropic effects (only on atrial strips) are obtained with acetylcholine

TABLE I. *pD_2-values for the positive inotropic effect on electrically paced atrial and ventricular cod heart strips, produced by cumulative doses of adrenergic drugs. Values are given ± S.E., numbers within parenthesis indicate number of experiments. Significant difference in potency compared to adrenaline is calculated: $p < 0.05$, $p < 0.01$, $p < 0.001$*

	Isoprenaline	*Adrenaline*	Noradrenaline
Atrial strips	8.19 ± 0.11 (8)	6.63 ± 0.19 (10)	5.84 ± 0.21 (9)
Ventricular strips	8.49 ± 0.39 (5)	6.94 ± 0.14 (6)	6.53 ± 0. 6 (9)

the cod. Ventricular strips were, however insensitive to acetylcholine, indicating the absence of cholinergic receptors for regulation of contraction force in the ventricle. This insensitivity to acetylcholine, as well as an absence of a cholinergic-inhibitory innervation, is also reported for the trout (*Salmo irideus*) and the eel (*Anguilla anguilla*) ventricles (Gannon 1971).

Contrary to the trout and plaice hearts (Gannon and Burnstock 1969 Cobb and Santer 1973), the cod heart reacted to stimulation of the vagi with a decrease in heart rate even with very low stimulation frequencies. A positive chronotropic effect of vagus stimulation was only obtained after atropinization. This effect, which is blocked by the β-adrenergic blocking agent propranolol, is probably due to release of catecholamines from adrenergic neurons in the vagi. The fluorescent histochemistry gives evidence for the presence of such neurons in the vagal cardiac branches. Adrenergic terminals are also found in the walls of the sinus venosus, the atrium and the ventricle, showing the existence of an adrenergic innervation. A functioning adrenergic excitatory innervation of the heart has previously been described in trout, *Salmo irideus* and eel, *Anguilla anguilla* (Gannon and Burnstock 1969 Gannon 1971).

The transient cardioacceleration often obtained after cessation of inhibitory vagal stimulation or after inhibition of the heart with acetylcholine might be due to a rebound phenomenon, similar to that described in the plaice heart by Cobb and Santer (1973). Pooling of perfusion fluid in the sinus venosus and duct of Cuvier during the inhibition of the heart activity might also influence the heart rate on cessation of this inhibition (Laurent 1962), although Johansen (1962) reports that variations in venous return does not affect the cod heart rate.

Cardiac acceleration could also be induced by stimulation of the fused 1st and 2nd spinal nerves, the blockade of this effect with propranolol indicates an action *via* adrenergic nerves. Fluorescent histochemistry also revealed the presence of such fibres in the branch of the first spinal nerves stimulated. The adrenergic fibres may reach the heart *via* the very fine nerves seen to leave the spinal nerve trunk and to enter the duct of Cuvier or the pericard in some of the dissected fish. Another possible pathway for the nerve fibres is *via* the ventral aorta and the bulbus arteriosus, which are heavily innervated by adrenergic nerves, and further into the cardiac tissue (Gannon and Burnstock 1969).

The adrenergic innervation to the heart thus seems to run partly in the vagi and partly in the first spinal nerves, the proportions being different from fish to fish. The lack of effect of stimulation of either one or both of the two types of nerves which was often obtained, can, however not be directly interpreted as a total lack of adrenergic innervation *via* that nerve. Damages of tissues during the preparation might interfere with the results. Neither were any of the nerves treated for fluorescence histochemistry completely devoid of adrenergic fibres, although it could not be determined whether these fibres reached the heart or not.

The adrenergic fibres reaching the heart *via* the vagi or the first spinal nerves are most likely of sympathetic origin, leaving the sympathetic chain at the ganglia in connection with the respective nerves (Nilsson 1976). Stimulation of the sympathetic chain also in some cases produced slight acceleration of the heart.

The effect of the adrenergic nerves is, at least partly direct on the β-adrenoceptors of the

Fig. 8. Inotropic effects of drugs on isolated strips from atria and ventricles, electrically paced at 1 Hz, 1 ms and 10 V and 0.5 Hz, 1 ms and 10 V respectively. Legend: ACh = acetylcholine, ADR = adrenaline, NA = noradrenaline, ATR = atropine. Arrows indicate addition of drugs. Agonists are added in cumulative doses, giving final concentrations in the bath of 10^{-8} M, 3 · 10^{-8} M, 10^{-7} M, 3 · 10^{-7} M etc. The tension is given in mp. Time bar 5 min.

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The conclusions from this study are that the cod heart receives a double antagonistic autonomic innervation. Like in other teleosts cholinergic inhibitory fibres, acting on muscarinic receptors, reach the heart via the cardiac branches of the vagi. The presence of an excitatory adrenergic innervation acting on β-adrenoceptors in the cod heart tissue is suggested from histochemical and pharmacological data. These fibres, which are most likely of sympathetic origin, run both in the vagi and in the fused 1st and 2nd spinal nerves, and appear to reach the sinus venosus and both atrial and ventricular tissue. Adrenergic neurons may also play a regulating role in the sinus venosus ganglion. Apart from the nerves, catecholamines released from the chromaffin tissue in the head kidney may have an important regulating capacity.

My thanks are due to Dr Stefan Nilsson for valuable and helpful criticism during this work. I also thank Mrs G. Rydgren, Mrs L. Utter and Mrs B. Vallander for their skilful technical assistance and Mr L. Hakansson for supplying fish. This work was supported by grants from the Swedish Natural Science Research Council and the Hierta-Retzius Foundation.

References

ABRAHAMSSON, T. and S. NILSSON, Phenylethanolamine-N-methyl transferase (PNMT) activity and catecholamine content in chromaffin tissue and sympathetic neurons in the cod, *Gadus morhua*. *Acta physiol. scand.* 1976. 96. 94–99.

BERKLEY, F. J. A possibility of sympathetic innervation of the fish heart. *Proc. Soc. exp. Biol.* (N.Y.). 1933. 31. 122–124.

BURNSTOCK, G. Evolution of the autonomic innervation of visceral and cardiovascular systems in vertebrates. *Pharmacol. Rev.* 1969. 21. 247–324.

CAMPBELL, G. Autonomic nervous systems. In: *Fish Physiology IV*. Hoar W. S. and D. J. Randall (Eds.) Academic Press, New York and London. 1970. pp. 109–132.

COBB, J. L. S. and R. M. SANTER, Electrophysiology of cardiac function in teleosts: Cholinergically mediated inhibition and rebound excitation. *J. Physiol.* (Lond.) 1973. 230. 561–573.

EHINGER, B., B. FALCK, H. PERSSON and B. SPORRONG, Adrenergic and cholinesterase-containing neurons of the heart. *Histochemie* 1968. 16. 197–205.

EULER, U. S. VON and R. FÄNGE, Catecholamines in nerves and organs of *Myxine glutinosa*, *Squalus acanthias* and *Gadus callarias*. *J. gen. comp. Endocrinol.* 1961. 1. 191–194.

FALCK, B., C. VON MECKLENBURG, A. MYHRBERG and H. PERSSON, Studies on adrenergic and cholinergic receptors in the isolated hearts of *Lampetra fluviatilis* (Cyclostomata) and *Pleuronectes platessa* (Teleostei). *Acta physiol. scand.* 1966. 68. 64–71.

FALCK, B. and CH. OWMAN, A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund.* 1965. Sect II. No 7. 1–23.

GANNON, B. J. A study of the dual innervation of teleost heart by a field stimulation technique. *Comp. gen. Pharmac.* 1971. 2. 175–183.

GANNON, B. J. and G. BURNSTOCK, Excitatory adrenergic innervation of the fish heart. *Comp. Biochem. Physiol.* 1969. 29. 765–773.

HOLMGREN, S. and S. NILSSON, Drug effects on isolated artery strips from two teleosts, *Gadus morhua* and *Salmo gairdneri*. *Acta physiol. scand.* 1974. 90. 431–437.

HOLMGREN, S. and S. NILSSON, Effects of some adrenergic and cholinergic drugs on isolated spleen strips from the cod, *Gadus morhua*. *Europ. J. Pharmacol.* 1975. 32. 163–169.

JOHANSEN, K. Cardiac output and pulsatile aortic flow in the teleost, *Gadus morhua*. *Comp. Biochem. Physiol.* 1962. 7. 169–174.

KULAEV, B. S. Nervous regulation of the rhythm of cardiac contractions in fish. Communication I. The effect of stimulation and section of the cardiac branches of the vagus on cardiac rhythm. *Bull. exp. Biol. Med.* 1957. 44. 731–734.

LAURENT, P. Contribution à l'étude morphologique et physiologique de l'innervation du cœur des Téléostéens. *Arch. Anat. micr. Morph. exp.* 1962. 51. Suppl.

heart muscle fibres. Specifically fluorescent varicose fibres are frequent in the atria, less common but existent in the ventricle. In both chambers the innervation seemed to be more dense in the outermost parts. This, however could be due to "shading" by the more intense autofluorescence in the rest of the tissue.

Part of the effect of the adrenergic nerves might be a regulation of the non-adrenergic (cholinergic?) ganglion cells in the sinus venosus. These are in fluorescence preparations seen to be surrounded by a network of adrenergic terminals. This has earlier been reported for mammals (Ehinger *et al*, 1968 Nielsen and Owman 1968).

The presence of a tonic activity in the nerves to the fish heart has been much discussed (McWilliam 1885 Von Skramlik 1935 Kulaev 1957 Rodinov 1959 Laurent 1962, Randall 1966) In this study atropine increases and propranolol decreases the heart rate of the perfused heart indicating a both cholinergic and adrenergic tonus in the preparations used. Whether this is due to a normal tonic activity of the nerves or to release and leakage from damaged nerve fibres has not been further investigated. The possibility of direct effects of the blocking agents can neither be completely dismissed, although low doses, especially of atropine, have been used. The effects of atropine and propranolol on the isolated strips might be due either to direct effects of the drugs or to blockade of the effect of transmitter leaking from the cut nerve endings

Stimulation of the nerves to the chromaffin tissue in the head kidney releases catecholamines, mainly adrenaline (Nilsson *et al* 1976) into the posterior cardinal vein. In the present experiments, where the outflow of perfusion fluid from the head kidney was allowed to pass through the heart, stimulation of the same nerves caused tachycardia. The effect could be blocked by propranolol and may therefore be regarded due to the above mentioned release of catecholamines. The effect on the heart was much bigger than the acceleration of the heart caused by direct nerve stimulation It can therefore be considered that release of catecholamines from the chromaffin tissue in the head kidney may play an important role in the regulation of the cod heart. This is contrary to the situation in mammals, where locally released neurotransmitters are found to be dominating in the control of the heart (see Rushmer 1962) It can also be noticed that the concentration range for the complete dose–response curve for adrenaline on the isolated cod atrial strips is 10^{-8} 10^{-4} M 0 18-1[illegible] μg/100 ml saline. This agrees well with the values of adrenaline content in blood samples from controls and stressed cod, 1 μg/100 ml and 4 μg/100 ml respectively obtained by Nilsson *et al* (1976) especially since it is most possible that the concentration of catecholamines reaching the heart directly from the head kidney *in vivo* is higher than the concentration in blood samples taken from the caudal vessels.

The effect of added adrenergic drugs has been difficult to abolish completely by washing out The catecholamines may somehow be retained in the heart tissue maybe in the catecholamine containing endothelial cells described in the cod atria (Sætersdal *et al* 1974), and from there slowly released.

In the perfused heart a positive inotropic effect of nerve stimulation or catecholamine release usually can be noticed only when the positive chronotropic effect is weak When the chronotropic effect is pronounced this may in the preparation used, due to limitations in the method mask an eventual inotropic effect

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Potentiation by Carbachol and Aminophylline of Histamine- and db-cAMP Induced Parietal Cell Activity in Isolated Gastric Glands

By

THOMAS BERGLINDH

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Abstract

BERGLINDH, T. *Potentiation by carbachol and aminophylline of histamine- and db-cAMP induced parietal cell activity in isolated gastric glands.* Acta physiol. scand. 1977. 99. 75–84.

The response to combinations of gastric acid secretagogues was studied in isolated glands from the rabbit gastric mucosa in terms of changes in oxygen consumption and accumulation of the weak base aminopyrine (AP). The latter reflects the acid secreting status of the glands. The following secretagogues were investigated: histamine, carbachol, aminophylline and db-cAMP. The histamine respiratory dose-response curve was shifted to the left in the presence of the phosphodiesterase inhibitor aminophylline. Both ED-50 and maximum response were significantly increased. Histamine-induced AP accumulation was also strongly enhanced by aminophylline ($5 \cdot 10^{-4}$ M). These results are consistent with the hypothesis that histamine stimulation of acid secretion is mediated by cyclic AMP. Carbachol-stimulated oxygen consumption could not be potentiated by aminophylline and the combined effect was only additive. The response to a combination of histamine and carbachol was a significant increase in oxygen consumption above what could be expected from an additive effect alone. Carbachol addition to glands prestimulated with histamine gave a rapid increase in the respiratory rate resulting in a new steady state level within 10–15 min, as compared with a time constant of about 40 min when both drugs were added simultaneously. Likewise AP accumulation increased more rapidly and reached a higher value after addition of histamine + carbachol as compared with histamine alone. The db-cAMP-stimulated oxygen consumption was in all respects equally affected by carbachol as was histamine stimulation. This indicates that the well known cholinergic potentiation of histamine stimulation is not due to an increased sensitivity of the histamine receptor but is of a more general nature. A mechanism involving intracellular availability of Ca^{++} is proposed as one possible explanation of this potentiation.

Key words: Gastric glands, histamine, carbachol, db-cAMP, aminophylline, oxygen consumption, aminopyrine

Through several investigations, the preparation of isolated glands from the rabbit gastric mucosa has proved to be a useful preparation for the study of acid secretory mechanisms in the mammalian parietal cell (Berglindh and Öbrink 1976, Berglindh et al. 1976, Berglindh 1976).

McWilliam, J A On the structure and rhythm of the heart in fishes with especial reference to the heart of the eel. *J Physiol* (Lond.) 1885 6 192 245

Nielsen, K. C. and Ch Owman Difference in cardiac adrenergic innervation between hibernators and non-hibernating mammals. *Acta physiol scand.* 1968. Suppl. 316.

Nilsson, S. Fluorescent histochemistry and cholinesterase staining of sympathetic ganglia in a teleost *Gadus morhua* *A ta Zool.* 1976. 57 69–77

Nilsson, S., T Abrahamsson and D J Grove, Sympathetic nervous control of adrenaline release from the head kidney of the cod *Gadus morhua*. *Comp Biochem. Physiol* 1976. In press.

Nilsson S. and D J Grove, Adrenergic and cholinergic innervation of the spleen of the cod, *Gadus morhua*. *Europ J Pharmacol* 1974 28 135–143.

Östlund E., The distribution of catecholamines I lower animals and their effect on the heart. *Acta physiol. scand* 1954. Suppl 112.

Randall, D J The nervous control of cardiac activity in the tench (*Tinca tinca*) and the goldfish (*Carassius auratus*). *Physiol Zool* 1966. 39 185–192.

Rodinov I M The reflex regulation of the activity of the heart in fishes. — I Reflex action on the heart from stimulation of the afferent fibres of the mesentery and intestine. *Bull. exp. Biol Med.* 1959 [illegible] 653–656.

Rossum, J M Van, Cumulative dose-response curves. II Technique for the making of dose-response curves in isolated organs nd the evaluation of drug parameters. *Arch. int Pharmacod.* 1963. [illegible] 299–330

Rushmer, R. F Effects of nerve stimulation and hormones on the heart the role of the heart in general circulatory regulation In *Handbook of Physiology* Section 2 Circulation Vol. I Hamilton, W. F nd P Dow (Eds.) American Physiol. Soc., Washington DC. 196[illegible] pp 533 550.

Saetersdal, T S., N P Justesen nd A W Krohnstad, Ultrastructure and innervation of the teleostean atrium. *J Molec Cell. Cardiol* 1974 6. 415–437

Santer, R. M. Ultrastructural and histochemical studies on the innervation of the heart of a teleost *Pleuronectes platessa* L. *Z Zellforsch.* 1972. 131 519–528

Schild, H O pA, a new scale for the measurement of drug antagonism. *Brit J Pharmacol.* 1947 [illegible] 189–296.

Skramlik, E. Von, Über den Kreislauf bei den Fischen. *Ergebn. Biol* 1935 2 1 130.

Yamauchi, A nd G Burnstock, An electron microscopic study on the innervation of the trout heart. *J comp Neur* 1968. 132 567 588

Young, J Z., On the utonomic nervous system of the teleostean fish *Uranoscopus scaber* *Quart J Micr scop Sci* 1931 74 491 535

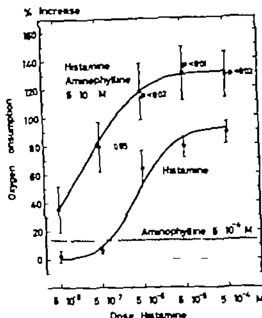


Fig. 1. Dose–response relationship between histamine concentration and oxygen consumption, with or without aminophylline 10^{-4} M. The vertical bars represent ± S.E. (n = 4). The basal gland oxygen consumption was 8.4 µl/mg dry wt. in 60 min. Aminophylline per se increased the respiration ( ). The p-values indicate whether the response to concomitant stimulation with histamine and aminophylline was significantly greater than the algebraic sum of the effects of the two drugs; p 0.05 is considered not significant ( ). The curves are drawn according to the integral of the normal probability curve obtained by probit analysis.

histamine response curve was shifted to the left, changing the ED-50 from 3.8 10^{-6} M to 2 10^{-7} M. Aminophylline alone increased the rate of respiration 13.3%. The histamine + aminophylline-induced increase in respiration was, however, significantly higher than the sum of histamine and aminophylline alone at histamine concentrations from 5 10^{-} M and higher. Since the maximum response was also significantly enhanced, the effects could be defined as a potentiation according to Cooke (1969) and Hirschowitz *et al.* (1973).

Carbachol and aminophylline. Carbachol (6.9 10^{-6} M) and aminophylline (5 10^{-4} M) alone or in combination were added to the gland suspensions and the oxygen consumption studied for 90 min. The percentage increase in oxygen consumption was (n = 5, mean ± S.E.): carbachol 24.7 ± 6.4%, aminophylline 9.0 ± 7.0%, carbachol aminophylline 36.1 ± 11.5%. Thus, in this type of study the combined response did not exceed a direct additive value.

Since, however, carbachol stimulation of isolated glands is of a transient nature with a peak value after approximately 15 min followed by a rapid decline (Berglindh *et al.* 1976), aminophylline was also investigated in that situation. The glands were preincubated with aminophylline 5 10^{-4} M and after a 15 min recording period, carbachol was added from the side-arm. As seen from the time–response curves in Fig. 2, basal respiration was stimulated by aminophylline, but the drug did not change the transient response after carbachol *per se*. Thus the phosphodiesterase inhibitor does not seem to potentiate carbachol stimulation or alter the transient course.

Histamine and carbachol. Histamine and carbachol alone or in combination were added to glands at the start of the incubation. The results are presented in Table I.

With a background of 4.5 10^{-6} M carbachol, histamine in increasing concentrations (up to 1.1 10^{-4} M) always significantly increased respiration above that which could be

Histamine was shown to be a potent secretagogue for the parietal cell in the isolated glands, and its actions were mimiced by db-cAMP (Berglindh *et al* 1976). In the present investigation the histaminecyclic AMP relationship was further studied by the use of the phosphodiesterase inhibitor aminophylline.

In previous investigations on the gastric mucosa, histamine has been shown to stimulate adenylate cyclase activity in all cases whereas simultaneously studied cholinergic drugs have produced divergent results, perhaps due to species differences (Nakajima *et al.* 1971 Perrier and Laster 1970, Ray and Forte 1974 Sung *et al* 1973). Therefore the glands' responses following carbachol were also investigated in the presence of aminophylline.

In the intact stomach there is a pronounced potentiating effect between histamine and cholinergic stimuli and also normally a cholinergic stimulatory tonus as a background (Andersson and Grossman 1965 Cooke 1969 Hirschowitz and Hutchison 1975). In contrast to *in vivo* conditions, where the effect on H -secretion is sustained, carbachol induces a transient response in the glandular preparation (Berglindh *et al* 1976). Thus in an attempt to approach *in vivo* conditions, the combined effect of histamine and carbachol was studied Finally also the influence of carbachol upon db-cAMP-stimulated glands was investigated

Materials and methods

Gastric glands. The glands were prepared from rabbit gastric mucosa according to the method of Berglindh and Öbrink (1976).

Chemicals. Carbamylcholine chloride (carbachol), N^6-O -d butyryl adenosine 3 5 -cyclic monophosphoric acid (db-cAMP grade II) as sodium salt and Aminophylline were obtained from Sigma Histamine di-hydrochloride was product of Schuchardt (Munich).

Aminopyrine (N-4-dimethyl-C14) with specific activity of 3.9 m Ci/mmol as purchased from NEN Chemicals.

Respiratory medium. NaCl 132.4 mM KCl 5 4 mM Na_2HPO 5 0 mM NaH_2PO 1.0 mM $MgSO_4$ 1 mM $CaCl_2$ 1.0 mM, phenol red 10 mg/l pH 7 4. Before use 2 mg/ml rabbit albumin (Sigma) and ml glucose were added.

Respiratory measurements The o ygen consumpti n was determined in a W rburg respirometer employing the same techniques as described by Berglindh and Öbrink (1976) and Berglindh *et al.* (1976). Either the drugs were added at the start of the incubation or from the side-arm of the Warburg flasks to preincubated glands. All drugs were dissolved in the respiratory medium nd added t obtai the desired concentration in 3 0 ml glandular suspension.

Aminopyrine (AP) accumulation. The theoretical and practical considerations concerning the use of the weak base aminopyrine for indirect measurements of acid production have been presented by Berglindh *et al.* (1976) and Berglindh (1976). In the present in estigation AP accumulation k netics were tudied by determinations of the AP concentration in the extragland lar water which ery clearly reflects the glandular uptake of AP as well as the accumulation ratio (AP in intraglandular water/AP i extraglandular water). Both methods were described by Berglindh (1976).

Results

Respiratory studies

Histamine and aminophylline Histamine was added in increasing concentrations to glands taken from the same preparation, with or without a simultaneous addition of aminophylline 5 10^{-4} M After 30 min preincubation the oxygen consumption was studied for 60 min. The dose-response curves are presented in Fig. 1 In the presence of [illegible]

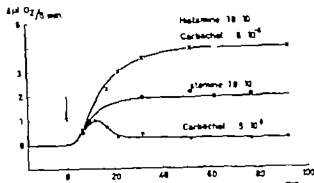


Fig. 3. The kinetic responses of glandular oxygen consumption to addition of carbachol, histamine and carbachol histamine. The drugs were added to preincubated glands as indicated by the arrow. The curves are drawn by eye. A representative example from series of 5 expts. is shown.

considerably higher than could be expected from any type of additive effect. In all expts. performed, however the combined response seemed to reach steady state later than did the histamine response.

Quite another type of response occurred when carbachol was added to glands prestimulated with histamine. The steady state histamine response increased almost instantaneously and reached new steady state level within 10–15 min. An example of such a response is presented in Fig. 4, which shows the difference between the histamine-induced respiration and the carbachol modulated one.

db-cAMP and carbachol. Two types of oxygen consumption studies were performed.

a) Db-cAMP (5 10^{-4} M) was added to glands prestimulated with carbachol (4.5 10^{-6} M) and the response was compared to that of db-cAMP alone. As seen in Fig. 5 the presence of carbachol enhanced the db-cAMP-response throughout the stimulatory course and gave steady state value, which was higher than the additive effect of db-cAMP and carbachol. The combined effect of db-cAMP and carbachol appeared, however to have a longer time constant than that of db-cAMP alone, before reaching steady state value. A simultaneous addition of carbachol and db-cAMP (compare method Fig. 3) gave a response, which was indistinguishable from that described above.

b) When, on the other hand, glands were prestimulated with db-cAMP (5 10^{-4} M)

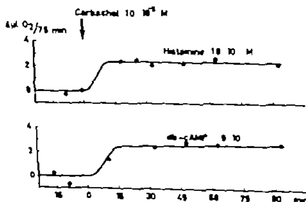


Fig. 4. Carbachol induced respiratory increase in glands prestimulated with histamine or db-cAMP. The figure show the difference between the carbachol stimulated preparations and their respective controls, stimulated by histamine and db-cAMP. The results are obtained simultaneously from the same gland population. The curves are drawn by eye. A typical example from series of 3 expts. is shown.

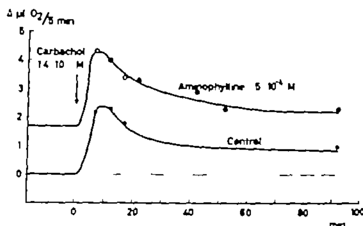


Fig. – The transient resp response after carbachol st effect of aminophylline upo stimulatory course. Carb was added as indicated t treated glands (control) in glands preincubated with a phylline. The curves are eye. Typical exampl of 4 expts. is shown.

expected from an additive effect alone. Linear transformation of the results gave for his a calculated V_{max} = 139 4 ± 9.2 % and ED-50 = 7 06 ± 1 61 10⁻⁴ M and for histam carbachol V_{max} = 179 4 ± 13.2 % and ED-50 = 3 14 ± 0.91 10⁻⁴ M (mean ± S.E.).

The effect of two other carbachol concentrations was also investigated in the pre of 1 1 10⁻⁴ M histamine. Carbachol 4 5 10⁻ M increased histamine-induced respir but not significantly above the calculated additive effect. A high concentration (1 4 10 gave a respiratory rate which was significantly lower than the direct additive one.

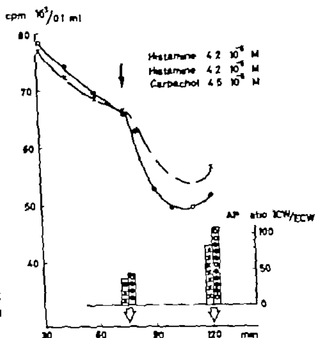
In the above expt. the rapid transient course following carbachol stimulation was encountered. This aspect was studied in kinetic expts. where the drugs were added to incubated glands. The response pattern obtained was compared with those of histamine carbachol alone. A representative expt. is shown in Fig. 3. Histamine and carbachol follo their "normal" stimulatory courses (Berglindh *et al.* 1976) whereas the combined stim tion displayed a much steeper increase and levelled off at a steady state value which

TABLE I. The effect of concomitant addition of histamine and carbachol on oxygen consumptio glands from the same populations. The table contains both the experimental and the theor (additive) results. The figures show mean ± S.E. (n = 4). The basal oxygen consumption 12.2 µl O_2/mg dry wt. in 90 min. p > 0.05 is considered not significant (s).

Histamine		Carbachol		Histamine + carbachol 4 5 10⁻⁴ M			
Histamine conc. (M)	Increase in oxygen con-sumption	Carbachol conc. (M)	Increase in oxygen con-sumption	Histamine conc. (M)	lncrease in oxygen con-sumption	Increase expected if additive	Difference found-expect
4.2 10⁻⁴	51.8 ± 1.3	4.5 10⁻	9 6 ± 2.2	4 + 10⁻⁴	107.5 ± 3 7	78.3	29.2 ± 4 7 p
1 1 10⁻⁴	84 1 ± 4.8	4.5 10⁻⁴	26.5 ± 2.6	1 1 10⁻⁴	135.3 ± 9 7	110 6	4 6 ± 7 5 p
1 1 10⁻⁴	130.9 ± 7 8	1 4 10⁻⁴	30.9 ± 2.0	1 1 10⁻⁴	174.4 ± 12.4	157 4	17 0 ± 5.3 p

Carbachol + histamine 1 1 10⁻⁴ M

Carbachol conc. (M)	% increase of oxygen con-sumption	Increase expected if additive	Difference found-expected
4.5 10⁻	156.1 ± 13.5	140.5	15.6 ± 7 5 n.s.
1 4 10⁻⁴	143.4 ± 6.5	161 8	− 18.4 ± 3 4 p < 0 02



g. 7 The effect of histamine and histamine + carbachol on AP accumulation determined from the change in AP concentration in the extraglandular fluid and the AP ratio. The latter was measured min before and 43 min after addition of the drugs as indicated by the bars. The concentration of glands was 2.5 mg dry wt/ml suspension.

In some expts. with higher aminophylline concentrations ($1 \cdot 10^{-3}$ *M*) a direct stimulatory influence upon accumulation was obtained. The results were, however divergent since aminophylline in these concentrations often increased the clumping of the glands (Berglindh *et al.* 1976).

Histamine and carbachol. Glandular AP accumulation in these expts. was followed by determination of the AP content in the extracellular fluid. The AP ratio was measured only at certain intervals. After 30 min preincubation, AP (0.4 µCi/ml) was added to each flask and the spontaneous accumulation studied for 45 min. Then histamine and histamine + carbachol were added in the concentrations seen in the example in Fig. 7. Following addition of the drugs, the AP uptake from the extracellular fluid was enhanced, and more so in the presence of the combined drugs than with histamine alone. This effect was also reflected in the higher AP ratio 45 min after addition.

The same experimental procedure as above for histamine and carbachol was applied to db-cAMP and carbachol.

The results indicated that carbachol may enhance the db-cAMP induced accumulation, but the effects were not as evident as for histamine.

Discussion

Previous studies on the stimulation-response coupling of the parietal cells in isolated gastric glands indicated a close relationship between histamine and cAMP-stimulation (Berglindh

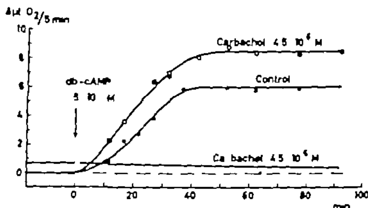


Fig. 5. The db-AMP ... respiratory increase in glands stimulated with carbachol. ... control db-cAMP was added ... untreated glands. The respir... rate with carbachol alone ... shown. The effect of carbachol per se (+0.2 μl O_2/5 min) throughout subtracted from the carbachol + db-cAMP curve, ... facilitates estimation of the ... of carbachol on the kinetics of the response. The curves are drawn by eye.

the addition of carbachol produced a rapid increase in oxygen consumption up to a new steady state level within 15 min. The results which are presented in Fig. 4 indicate furthermore the similarity between the histamine- and the db-cAMP responses following carbachol addition.

Aminopyrine accumulation

Histamine and aminophylline The influence of aminophylline on histamine-induced aminopyrine accumulation was studied by determination of the aminopyrine (AP) ratio (ICW/ECW) at each point. AP 0.4 μCi/ml was added to two flasks containing glands from the same population. After 45 min incubation aminophylline 5 10^{-} M was added to one flask and the effects studied for another 30 min. Histamine 5 10^{-6} M was then added to both flasks and the sampling continued for a final 45 min period. The results of a typical experiment are presented in Fig. 6. The aminophylline concentrations used in these investigations did not appreciably affect the accumulation ratio, as shown in the figure. Upon addition of histamine, however, the aminophylline treated glands responded much more rapidly and reached a higher maximal accumulation than did the histamine control.

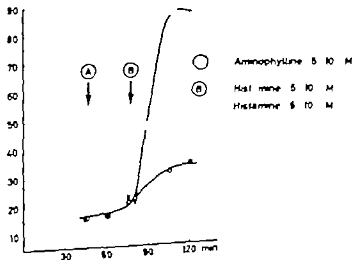


Fig. 6. Influence of aminophylline on histamine induced AP accumulation determined ... AP ratio (ICW/ECW). The AP was added from the start. Aminophylline ... added to one flask ( ) at A. The curves, however, followed the same course until histamine was added to both flasks at B. The concentration of glands in each flask ... 7.9 mg dry wt./ml suspension.

Fig. 7 The effect of histamine and histamine + carbachol on AP accumulation determined from the change in AP concentration in the extracellular fluid and the AP ratio. The latter was measured just before and 45 min after addition of the drugs as indicated by the bars. The concentration of glands was 3 mg dry wt/ml suspension.

In some expts. with higher aminophylline concentrations ($1-2 \cdot 10^{-4}$ M) a direct stimulatory influence upon accumulation was obtained. The results were, however, divergent since aminophylline in these concentrations often increased the clumping of the glands (Berglindh *et al.* 1976).

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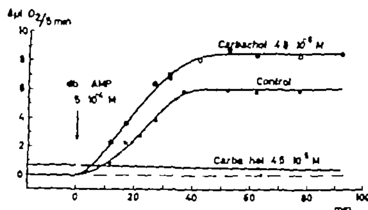


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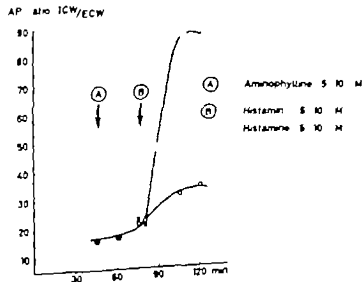


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965). The denervation effect could be abolished by a small amount of urecholine (Hirschowitz and Hutchison 1975). Thus it appears that histamine-induced secretion *in vivo* is normally supported by cholinergic background which might serve as a modulator of the acid secretory rate. When carbachol was added to glands prestimulated by histamine (Fig 4), there was an almost instantaneous increase in the respiratory response up to a new steady state level. This rapid effect is in accordance with a modulator role for cholinergic stimulation. Since the simultaneous addition of histamine and carbachol induced an oxygen consumption which in fact reached its steady state later than histamine alone, a secreting parietal cell is thus a prerequisite for the rapid durable carbachol effect.

The potentiating effect also to some extent appeared to be dependent on the carbachol concentration. Thus high doses of carbachol were less effective and in fact gave values below those expected even from an additive effect alone. This is in agreement with findings in vivo (*cf* Hirschowitz and Hutchison 1975), where the combined use of high concentrations of histamine and carbachol decreased the acid response.

Several suggestions have been put forward to explain the potentiating effect of carbachol on histamine stimulated H secretion. It has generally been proposed that cholinergic stimulation sensitizes the parietal cell, or more specifically the histamine receptor towards histamine. Recently Hirschowitz and Hutchison (1973) suggested that potentiation (increased V_{max}) between histamine and acetylcholine could be explained by postulating spare cholinergic receptors and that the synergism (increased effect of histamine) was due to an increased sensitivity of the histamine receptor. The finding in the present work that db-cAMP-or histamine-induced respiratory responses were equally affected by carbachol is, however not consistent with an activation of the histamine receptor since db-cAMP exerts its stimulatory effect intracellularly.

A possible hypothesis for the potentiating effect of cholinergic stimulation might involve an influence of Ca the importance of which has been shown for several other secretory systems. Accordingly cAMP has been shown to release Ca from intracellular stores and acetylcholine, some tissues, to increase the cell membrane permeability to Ca^{++} and often in a transient way (for Review see Berridge 1975).

Exhorn *et al.* (1973) showed that vagal stimulation or acetylcholine infusion increased the cyclic GMP content in canine gastric mucosa in a rapid transient way. This is of interest since the only know activator of guanylate cyclase is an increase in intracellular Ca (*cf* Berridge 1975). Although the cellular localization of the cGMP increase was not determined, that result may indicate that cholinergic stimulation could be mediated by an increase in free intracellular Ca.

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References

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et al 1976) The present results show that the phosphodiesterase inhibitor aminophyllin potentiates histamine-induced respiratory responses, in terms of significant increases both i ED-50 and in maximal response (V_{max}) This effect was also confirmed in the AP accum lation studies, where a potentiation of the acid secretory mechanism was revealed The findings fulfil one of Sutherland's criteria for a hormone-cyclic AMP relationship i regard to histamine Additional support for such a coupling is that exogenous db-cAM (and cAMP) mimics the effects induced by histamine in the isolated gastric glands (Berglind *et al* 1976, Berglindh 1976) and that histamine also activates adenylate cyclase from rabb gastric mucosa (Sung *et al* 1973). It thus seems very probable that histamine-stimulate acid secretion in the rabbit is mediated by cAMP

In *in vivo* studies on rodent gastric mucosa, an increase in cAMP content has also bee shown after stimulation with cholinergic drugs or pentagastrin (Bersimbaev *et al* 1971 Narumi and Maki 1973 Rouff and Sewing 1974) *In vitro* no increase in the cAMP pro duction with pentagastrin or carbachol in guinea pig gastric tissue was obtained (Karppane *et al* 1974) neither did these agents stimulate adenylate cyclase from rabbit gastric mucos (Sung *et al* 1973). The inability of aminophylline to potentiate the response to carbacho in the isolated glands are thus in agreement with other *in vitro* reports. Previously penta gastrin was shown to be without stimulating effects upon the isolated gastric glands (Berg lindh *et al* 1976) and the presence of aminophylline did not alter this negative response (Berglindh unpublished results)

In a "reduced" system like isolated gastric glands only the direct response to drugs should be obtained (Berglindh and Öbrink 1976, Berglindh *et al* 1976). Since pentagastrin and cholinergic drugs stimulate the production of cAMP *in vivo* but not *in vitro* it may be suggested that they act partly via histamine, which appears equally active regardless of the type of preparation Gastrin and pentagastrin are potent stimulators of histamine release from the mammalian gastric mucosa (*cf* Kahlson *et al* 1973) and such an effect of both pentagastrin and cholinergic stimuli has been shown in the amphibian gastric mucosa (Kasbekar *et al* 1969 Rangachari 1975) Very recently Lundell (1976) found an increase in the histamine forming capacity of the gastric mucosa in antrectomized rats following central vagal stimulation or distension of the stomach.

In vivo it is well established that histamine and cholinergic stimuli (vagal- or drug-induced) do interact and potentiate hydrochloric acid secretion (Andersson and Grossman 1965 Cooke 1969 Hirschowitz and Hutchison 1975). This relationship was also found in the isolated gastric glands, where a significant increase in oxygen consumption was recorded, above that which could be expected from an additive effect alone. In the kinetic studies when both drugs were added simultaneously the increase obtained both in oxygen consumption and in AP accumulation was more rapid and reached a higher level, than each drug alone could produce. Carbachol alone induced a transient increase in both parameters with a peak value after approximately 15 min (Berglindh *et al* 1976, Berglindh 1976) but in the presence of histamine a sustained effect was obtained. This may indicate that a "normal" cholinergic responce is dependent on the presence of histamine (Berglindh *et al* 1976).

In the dog, vagal denervation of the stomach decreases its sensitivity towards histamine or pentagastrin, without altering the maximal histamine response (Andersson Grossman

1965). The denervation effect could be abolished by a small amount of urecholine (Hirschowitz and Hutchison 1975). Thus it appears that histamine-induced secretion *in vivo* is normally supported by a cholinergic background which might serve as a modulator of the acid secretory rate. When carbachol was added to glands prestimulated by histamine (Fig. 4), there was an almost instantaneous increase in the respiratory response up to a new steady state level. This rapid effect is in accordance with a modulator role for cholinergic stimulation. Since the simultaneous addition of histamine and carbachol induced an oxygen consumption which in fact reached its steady state later than histamine alone, a secreting parietal cell is thus a prerequisite for the rapid durable carbachol effect.

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References

ANDERSSON, S. and M. I. GROSSMAN, Effect of vagal denervation of pouches on gastric secretion in dogs with intact or resected antrums. Gastroenterology 1965, 48, 449–462.

BERGLINDH, T Effects of common inhibitors of gastric acid secretion on secretagogue induced respiratio and aminopyrine accumulation in isolated gastric glands. *Biochim biophys. Acta* (Amst.) 1976 In pre
BERGLINDH, T and K. J ÖBRINK, A method for preparing isolated glands from the rabbit gastric muco *Act physiol scand.* 1976, *96* 150–159
BERGLINDH T H F HELANDER and K J ÖBRINK, Effects of secretagogues on oxygen consumptio aminopyrine accumulation and morphology in isolated gastric glands. *Acta physiol. scand.* 1976 *9* 401–414
BERRIDGE, M J The interaction of cyclic nucleotides and calcium in the control of cellular activity I *Advances in Cyclic Nucleotide R search* Vol. 6. Ed. P Greengard and G A Robinson, Raven Pre New York 1975 1 98
BERSIMBAEV R I S. V AROUTINSKAYA and R. I SALGANIK The stimulating action of gastrin pentapepti and histamine on adenyl cyclase activity in rat stomach *Experientia* (Basel) 1971 *27* 1389–1390.
COOKE, A R Potentiation of acid secretion in dogs. *Amer J Physiol* 1969 *216* 968–973
EICHHORN J H E. W SALZMAN and W SILEN, Cyclic GMP in stimulated gastric mucosa. *Surgical Foru* 1973 *24* 363–365.
HIRSCHOWITZ, B. I and G A HUTCHISON, A working hypothesis for urecholine effects on histam stimulatio of gastric secretion. *Scand J Gastroent* 1973 *8* 569–576.
HIRSCHOWITZ, B I nd G A. HUTCHISON, Effects of vagotomy on urechol ne-mod fied histamine do responses in dogs. *Amer J Physiol* 1975 *228* 1313–1318.
HIRSCHOWITZ, B. I G SACHS and G HUTCHISON, Lack of potentiation or synergism bet een histamin and pentagastrin in fistula dog. *Amer J Physiol* 1973 *224* 509–513
KAHLSON G E. ROSENGREN and S. E SVENSSON, Histamine and gastric secretion with special referen to the rat In *I t Ency l Pharmacol Ther* Section *39 ( ). Vol 1* Pharmacology of gastrointestina motility a d secretion Ed. P Holton Pergamon Press. 1973 41 102.
KARPPANEN, H O P J NEUVONEN P R BIECK nd E. WESTERMANN, Effect f histam ne, pentagastri and theophylline on the production of cyclic AMP i isolated gast ic tissue of the guinea pig *Naun Schmiedeberg s Arch Pharmac* 1974 *284* 15–23.
KASBEKAR D K. H A. RIDLEY and J G FORTE, Pentagastri and acetylcholine relation to histamine i H secretion by gastric mucosa. *Amer J Ph siol* 1969 *216* 961 967
LUNDELL, L., The role of cholinergic excitation the formation of hist mine w thin *the rat gastric mucos Acta ph iol scand* 1976. *97* 145–149
N KAJIMA, S., B I HIRSCHOWITZ and G SACHS, Studies on adenyl cyclase in nect rus gastric mucos *Arch Biochem.* 1971 *143* 123–126.
NARUMI, S. and Y M I, Possible role of cyclic AMP ga tric acid secretion in rat Activ tion of carboni anhydrase *Biochim. bioph s. Act* (Amst) 1973 *311* 90–97
PERRI R C. V and L. LASTER, Adenyl cyclase activity f guinea pig gastric mucosa st mulation by histamin nd prostaglandins. *J lin Invest* 1970. *49* 73 a.
RANGACHAR P K Histam ne release by gastric st mulants *Nature* (Lond) 1975 *253* 53 55
RAY T K. and J G FORTE Adenyl cyclase f yotic cells. It association with different cell lar mem branes. *Biochim. biophys A ta* (Amst) *1974 320 320–339*
ROUFF H J a d K F SEWING, R t gastric mucosal cAMP following chol nergic and h stam ne stimula tion *Europ J Pharmac* 1974. *28* 338 343
SUNG, C. P B C. JENKINS, L. R BU NS, V H CKNEY J G SPENNEY G S CHS and V D WIEBELHAUS, Adenyl and guanyl cyclase i rabbit gastric mucosa *Amer J Ph siol* 1973 *225* 1359 1363

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From the Laboratory for the Theory of Gymnastics, and August Krogh Institute, University of Copenhagen, Denmark

Regulation of Respiration: "The Black Box"

By

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Abstract

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A close relation between the state of wakefulness, the electroencephalogram, and the respiration has been demonstrated (*Bülow* 1963). In the present paper it was assumed that as opening and closing of the eyes influence the EEG and thus probably the state of wakefulness, the responsiveness of the respiratory center may likewise be influenced by the simple act of closing the eyes. Seven subjects were studied in the steady state of rest breathing ambient air, 4–5% CO_2 in air, or 11% O_2 in nitrogen. In experimental periods of 4–5 minutes their pulmonary ventilation and alveolar P_{CO_2} were measured while they randomly were either reading a book, or were blindfolded. It was found that in all three conditions closing of the eyes resulted in a mean decrease of $\dot{V}$ of 8 to 14% with a concomitant increase of P_{ACO_2} of about 1 mmHg. It is tentatively suggested that other and stronger central or peripheral stimuli impinging on the reticular formation of the brain may explain the changed sensitivity of the respiratory center towards the chemical stimuli in conditions like exercise, chronic hypoxia etc.

In most studies of respiratory regulation, known or presumed stimuli are fed into the respiratory center (the "black box" of e.g. Defares (1964)) via the blood stream, the cerebrospinal fluid or via nervous reflex pathways, and the outcome is assumed to be the direct response to these stimuli. Several models and equations describing this relationship have been worked out (e.g. Gray (1950), Grodins *et al.* (1954), Lloyd and Cunningham (1963), Cunningham (1974)). Most of these models, however, have exemptions where they are not applicable, as for instance in muscular exercise, in sleep, and during acclimatization to altitude (hypoxia). In these cases changes in the known respiratory stimuli do not elicit the predicted responses and the changes in ventilation cannot be explained as caused by these stimuli. It is an obvious thought that this may be because changes have taken place in the "black box"—the respiratory center. Such changes have earlier been described using the words "sensitivity of the respiratory center", "responsiveness" etc. (Lindhard (1911), Marius Nielsen (1936), [illegible]), but the expression has for several reasons fallen into misscredit, partly because also sensitivity changes needed an explanation. The modern view of the respiratory center as an integrated part of the reticular formation of the brain stem with the subsequent possibilities

of influencing this part of the brain via both higher cerebral and peripheral ascending p ways provides renewed background for speaking of the sensitivity of the respiratory cer A cerebral drive responsible for the often observed continuation of ventilation after volun hyperventilation with below threshold P_{CO_2} values, was postulated by e.g. Fink (19 and ascending proprioceptive or chemoceptive nerve impulses from exercising or mov limbs in awake man have repeatedly been ascribed the role of increasing the sensitivity the respiratory center towards CO or H (e.g. Lindhard 1911 Nielsen 1936, Asmu 1973) Electroencephalography (EEG) makes it possible to get an objective signal of state of wakefulness of the brain Bülow (1963) made extensive use of this method and dem strated a clear correlation between various stages of EEG and respiration during wakefuln drowsiness and sleep

Changes in EEG are seen in awake subjects when they close their eyes (Berger 19: Supposing that this signals a changed state of arousal in the CNS, including the respirat center it was the purpose of the present paper to investigate possible changes in the rest ventilation under two conditions of awake rest 1) resting in a reclining chair while readin book and 2) resting in the same position but with closed eyes, blindfolded

Methods and procedure

The pulmonary ventilation was measured as V_E from expired air collected in Douglas bags. The b were emptied under standard low suction into a well balanced Tissot gasmeter Samples for anal were taken i well greased syringes and analyzed within 2 to 3 h Inspired air was either room air mixtures containing 4–5% CO in air o 10–11 O in nitrogen. The gas mi tures were administe through a large Douglas bag, maintained half f lled by manipulation of the valves on the gas cylin and contai ing some liters of water to moisten the air All gases were analyzed on the Scholander apparat The subjects were fitted o t with nose clip and breathed through a low resistance Engboff val e. A thermis in the valve registered the temperature fluctuations which were recorded on a inkwriting recorder w known paper speed. From the recordings the respiratory frequency was measured for use with the determina tion of V_T. From the ventilatory data and the gas samples the mea alveolar gas tensions could be c culated by means of the Bohr formula and a dead space estimated from the size of V_T (Asmussen and Nieh 1956).

All expts. were perf rmed in the morning i a quiet room. The subjects rested in a comfortable low ch for bout 45 min. In the airbreathing expts. they then were f tted-out with noseclip and mouthpiece, a the next 10–15 min were used t flush the bags with expired ir and empty them again. When gas mixtu were used the gas was administered for period of 20–25 min, includ ng the time used i flushi g the ba The actual expt. was then begun. O each xperimental day 6 continuous sampling periods, each of 4 min duration were used In 3 of these periods the subject was readi g a book duri g the expt In the oth three he/she was blindfolded with closed eyes. Periods with open and w th closed eyes were mixed random and there was no pause between the periods. The number of test period fo each cond tio nd for ea subject are given i parentheses i col mns 2, 3 6, 7 10 nd 11 of T ble I

As subjects served 5 men, aged 26 to 41 years, nd 2 women, 27 nd 32 years old All were healthy an physically fit, and accustomed to respiratory proced res. One of the men knew the purpose of the stud but the 6 other subjects were uninformed nd did not guess the purpose.

The use of gas mixtures was t first introduced because it w ntended t tudy the effect of openi g a closing the eyes on the CO_2 response curves, as it was expected that potentiati g or add tive effects might b detected. As it turned out, individual and day-to-day variations of V_E nd P_{ACO_2} were so la ge that suc effects—if present—could not be seen. For the same reason the V_E with closed eyes, mean of 3 from on day will be presented as a percentage of the mea of 3 V_E with open eyes of the same day instead of a bsolute differences.

TABLE I Changes in resting respiration on closing the eyes

Subject	Breathing air				Breathing 4–5 % CO_2 in air				Breathing 11 % O_2 in nitrogen			
	V_E(BTPS) l min⁻¹		%	ΔP_{ACO_2}, mmHg	V_E(BTPS) l min⁻¹		%	ΔP_{ACO_2}, mmHg	V_E(BTPS) l min⁻¹		%	ΔP_{ACO_2}, mmHg
	Eyes op. I	Eyes cl. II	100 II/I	II–I	Eyes op. I	Eyes cl. II	100 II/I	II–I	Eyes op. I	Eyes cl. II	100 II/I	II–I
C B R, m	6.7 (6)	5.3 (8)	79	1.1	38.5 (16)	30.5 (24)	79	+0.4	—	—	—	—
T O f	—	—	—	—	16.2 (6)	15.6 (6)	96	+0.1	8.1 (5)	7.6 (6)	94	−0.9
B W f	—	—	—	—	26.8 (8)	24.4 (8)	91	+2.7	7.9* (9)	6.2 (9)	78	+4.4
H T m	7.6 (3)	6.8 (3)	89	+0.8	—	—	—	—	8.6 (15)	8.1 (15)	94	+0.2
G H m	7.8 (6)	6.4 (6)	82	0.5	23.5 (9)	22.0 (9)	94	+1.2	13.0 (18)	10.4 (9)	80	+0.2
I R m	—	—	—	—	30.2 (3)	24.9 (3)	82	+0.9	12.0 (9)	11.6 (9)	97	−0.3
A B m	12.1 (8)	11.5 (7)	95	2.7	29.8 (6)	28.3 (8)	95	+0.5	16.6 (14)	14.6 (14)	88	+0.7
Weighted means			85.4	+1.1			92	+1.1			89.6	+0.9
			8				12				24	
Se			1.40				1.82				2.00	

* Difference between values significant on 0.025 level.

Results

Table I shows the average absolute values of V for each subject in the three different conditions of inspired air with open and with closed eyes. A Student's t-test performed on each individual's absolute data showed that only the data marked with an asterisk were significantly different (p 0.025). This is due to the wide scattering of the day-to-day expts., a scatter which to some extent may be due to small day to day variations in the composition of the gas mixtures, when such were used as inspired air. The average percentage values (Table I) calculated as mentioned above were also individually different and varied for the same subject from one condition to another.

The weighted means of all ratios between V s with closed and open eyes are also presented in Table I, with their standard error of the mean. It can be seen that all of them are significantly different from 100.

P_{ACO_2} (Table I) showed on an average an increase of about 1 mmHg under all three conditions when the subjects closed their eyes and V decreased. Individually there was 1 subject in the air expts. and 2 subjects in the low O_2-expts. who showed decrease in P_{ACO_2}. In the two latter cases the absolute values of P_{ACO_2} were about 30 mmHg and 23 mmHg, respectively—i.e. presumably below the threshold value for P_{CO_2}.

In spite of the 20 min pre-period when breathing gas mixtures, the nitrogen had not quite equilibrated. Accurate measurements of V_{O_2} consequently could not be made under these

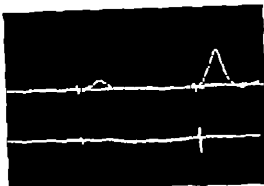
conditions. But in the air expts. it was found that V_{O_2} often declined slightly when the e
were closed. In our best studied subject (A B.) it remained unchanged, and in his case
$\dot{V}_E/\dot{V}_{O_2}$ was found to decrease from 25 6 1/1 to 24 2 1/1

Discussion

The present expts. have shown that closing of the eyes in the resting state is accompar
by a mean reduction of V_E of 8 to 14%. These changes are small compared to the d
variations in V_E, but taken all together they are statistically significant. Also the accompa
ing changes in P_{ACO_2} (an average increase of about 1 mmHg), and in V_E/V_{O_2} (measured c
in air) supports the finding of a decreased V_E when the subjects have closed their e
The problem raised in the introduction, that the state of wakefulness or arousal has a di
effect upon the response of the respiratory center to the two stimuli P_{CO_2} (or H) a
hypoxic P_{O_2} *thus seems to be affirmed.*

The assumption that opening and closing of the eyes change the state of wakefulnes
based upon the well known effect on the EEG reported in the literature (Berger 1929 a
This change involves several autonomous and reflex functions besides the respirat
In the present investigation it was for instance found that the briskness of a simple myot
reflex, the patellar reflex, was clearly correlated to the subjects opening or closing the e
In subject A B. the average of 16 repeated standardized taps on the tendon gave a mechani
response of 1 75 arbitrary units, when the eyes were open as opposed to 0 54 when the e
were closed Fig. 1 shows an example with both mechanical and electromyographic respon
represented In Bülow s (1963) expts. it was clearly established that changes in the EE
from stage A (wakefulness) to stage B (drowsiness) at the beginning of sleep, was follow
by changes in $\dot{V}_E$, and in the shape of the V_E/CO response curve. The response curve shif
to the right and usually showed a less steep slope, $\Delta V_E/\Delta P_{ACO_2}$. In the present expts. it w
not possible to obtain CO_2-response curves with a reproducibility sufficient to make c
whether similar changes accompanied opening/closing of the eyes in the awake state. T
average increase of about 1 mmHg in P_{ACO_2} with closed eyes, however points to a rig
shift of the response curve, but no systematic variation in the slope of the curve was fou
In hypoxia all P_{ACO_2} values were lowered some of them so much (down to 23 mmH
that the threshold value of P_{CO_2} as a respiratory stimulus had been passed This may be t
reason for the more irregular changes in P_{ACO_2} on closing the eyes in this condition E
the expts. show that also with hypoxia as "the sole stimulus V_E was smaller with clos
eyes.

The "change of sensitivity" of the respiratory center towards CO and hypoxia demo
strated in the present expts. may well be an extreme example of what takes place wh
other more pronounced changes in the state of wakefulness take place. Besides sleep (fo
references see Bülow (1963)), morphia and other drugs are known to *decrease* the "sensitivity
of the center (Lindhard 1911) Bülow whose awake state (stage A) was maintained wi
closed eyes, further studied a stage A_s called "tense alertness" in which his subjects ha
open eyes and were alerted e.g. by having to solve problems mentally and he actual
found an increased ventilation as compared to stage A (with closed eyes) The most powerf



1 Patellar reflex, left with closed eyes, with open eyes. Top: mechanograms. ... electromyograms. Subject A. B.

or for increasing the sensitivity of the respiratory center is probably muscular exercise, ...ly acting through incoming signals from the exercising and moving limbs (Asmussen, ...sen and Wieth-Pedersen (1943), Kao *et al.* (1955), Dejours *et al.* (1964)), possibly also an increased "cortical excitatory state". The two influences—peripheral and central—...y well be present at the same time and the central part need not necessarily be caused by ...adiation of motor impulses (Krogh and Lindhard (1913)), but may come from other ... sensory parts of the brain. The fact that electrically induced work has the same effect ... ventilation as voluntary work (Asmussen *et al.* (1943)) thus may be explained by an ...reased arousal due to sensory signals from the stimulated muscles.

Nielsen (1936) showed that acclimatization to hypoxia caused a gradual increase in ... above the immediate chemoreceptor response to low P_{aO_2}, and Forster *et al.* (1974) ...owed that this secondary rise in V_{O_2} was not caused by measured humoral factors in ...ood or CSF and suggested an increased sensitivity or responsiveness of the respiratory ...nter caused by increased CNS arousal. This suggestion was supported by experiments ...th drugs (Doxapram), and with the "visual evoked response" and EEG (Forster *et al.* ...975)).

The general observation that respiration may be influenced by "the surrounding traffic ...f activity" through the reticular formation (Burns (1963)) thus is not new. Neither is the ...bservation that opening and closing the eyes influences the CNS state of arousal. The ...resent expts. emphasize that respiration is not only regulated by the accepted stimuli ...P_{CO_2}, [H$^+$] and P_{O_2} of blood or CSF but that the state of "the black box" into which these ...timuli are fed may play a dominant part in what comes out of it again. Theoretically ...ll sensory and intra-encephalic signals that impinge on the reticular formation thus may ...lay ... role in the regulation of respiration.

References

ASMUSSEN, E., Ventilation at transition from rest to exercise. *Acta physiol. scand.* 1973, *89*: 68–78.

ASMUSSEN, E. and M. NIELSEN, Physiological dead space and alveolar gas pressures at rest and during muscular exercise. *Acta physiol. scand.* 1956, *38*: 1–21.

ASMUSSEN, E., M. NIELSEN and G. WIETH-PEDERSEN, Cortical or reflex control of respiration during muscular work. *Acta physiol. scand.* 1943, *6*: 168–175.

BERGER, H (1929) cit. from *Gray IV* Intrinsic rhythms of the brain, in Hb. of Physiol. Section I Neurophysiol., Washington D C. *Amer Physiol. Soc* 1959 279–298.
BÜLOW K Respiration and wakefulness in man. *Acta physiol scand* 1963, 59 Suppl. 209
BURNS, B. D The central control of respiratory movements. *Brit Med. Bull.* 1963, 19 7 9
CUNNINGHAM, D J C., *Integrative aspect of the regulation of breathing personal view* In. Respir Physiol Guyton and Widdicombe, eds. Physiol. Series 1 vol. — MTP Intern. Rev Science. London, Butterworth 1974, 303–370
DEJOURS, J G Principles of feedback control and their application to the respiratory control system In Hb. of Physiology Section 3 Respiration, vol. 1 Eds. Fenn, W O and H. Rahn. Washington DC *Amer Physiol Soc* 1964 649–680
DEJOURS, P Control of respiration in muscular exercise. *Amer Physiol Soc* 1964. 631–648.
FINK, B R., Influence of cerebral activity in wakefulness on regulation of breathing *J appl. Physiol* 1961 *16* 15–20.
FORSTER, H V J A. DEMPSEY E. VIDRUK and G Do PICO, Evidence of altered regulation of ventilation during exposure to hypoxia. *Respir Physiol.* 1974 *20*, 379–392.
FORSTER, H V R. J SATO, J K. DEMPSEY and M J HORKO, Effects of sojourn at 4300 m altitude electroencephalogram and visual evoked response. *J appl Physiol.* 1975. *39* 109–113
GRAY J S., *Pulmonary ventilation and its physiological regulation.* Springfield, Ill. Thomas Publ. 1950 *p* 82.
GRODINS, F S., Analysis of factors concerning the regulation of breathing in exercise. *Physiol. Rev* 1950 *30*. 220–239
KAO, F F B B SCHLIG and C. McC. BROOKS, Regulation of respiration during induced muscular work in decerebrate dogs. *J appl Physiol.* 1955. *7* 379–386.
KROGH, A. and J LINDHARD The regulation of respiration and circulation during the initial stages of muscular work *J Physiol.* (Lond.) 1913 *47* 112 136.
LINDHARD J On the excitability of the respiratory centre. *J Physiol.* (Lond.) 1911 *42*. 337 358.
LLOYD B. B and D J C. CUNNINGHAM, *A quantitative approach to the regulation of human respiration* In The regulation of human respiration, eds. Cunningham and Lloyd, Oxford Blackwell 1963, 331 349
NIELSEN M Untersuchungen über die Atemregulation beim Menschen, besonders mit Hinblick auf die Art des chemischen Reizes. *Skand. Arch. Physiol.* 1936. *74* Suppl. 10. 84–208.

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Time Course of Changes in Human Skeletal Muscle Succinate Dehydrogenase and Cytochrome Oxidase Activities and Maximal Oxygen Uptake with Physical Activity and Inactivity

By

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Abstract

HENRIKSSON, J and J S. REITMAN. *Time course of changes in human skeletal muscle succinate dehydrogenase and cytochrome oxidase activities and maximal oxygen uptake ith physical activity and inactivity* Acta physiol. scand. 1977 99 91 97

Succinate dehydrogenase (SDH) and cytochrome oxidase activities in the lateral vastus of the human quadriceps femoris muscle together ith total body $\dot{V}_{O_2}$ max were followed during an 8–10 week period of endurance training (13) and successive 6 week period without training (8) During the training period there was gradual increase in both V_{O_2} max and muscle oxidative enzyme activities, all being significantly different from the pre-training levels after 3 eeks of training. After 8 weeks of training V_{O_2} max was 19 %, vastus lateralis SDH 32 %, and cytochrome oxidase activity 35 % above the pre-training levels respectively 6 eeks post-training V_{O_2} max was still 16 above the pre-training level, and not significantly different from the level at the end of training (p 0.2) I contrast vastus lateralis SDH activity had returned to the pre-training level. Cytochrome oxidase activity had returned to the pre-training level than two eeks post-training. The significantly faster post-training decline in skeletal muscle oxidative enzyme activities in contrast to that of the V_{O_2} max indicates that an enhancement of the oxidative potential in skeletal muscle not necessary for high V_{O_2} max. Moreover the fast return to the pre-training level of both SDH and cytochrome oxidase activities indicate high turnover rate of enzymes in the TCA cycle as well as the respiratory chain

It is ell documented that the maximal oxygen uptake as well as the activity of oxidative enzymes in skeletal muscles varies with the level of physical activity (for ref see Holloszy 1975) However there exists very little information about the time course of these changes as well as how closely they are correlated with each other

By comparing the temporal sequence of different adaptive changes it may be possible to obtain some indications about the regulatory mechanisms of the adaptive processes. One example of this approach is given in the report by Brown *et al.* (1973), showing that during long-term electrical stimulation of a fast muscle of rabbit by frequency naturally

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occurring in a nerve to a slow muscle, growth of new capillaries appears after 4 days ar precedes the changes in the activity of oxidative enzymes by 10 days This informatc suggests that enhanced blood supply and thus oxygen delivery to the muscle may be requ for a stimulation of the synthesis of oxidative enzymes.

The purpose of the present study was to follow changes in skeletal muscle oxidative enzy activities and relate these to the variation in maximal oxygen uptake during a period enhanced physical activity followed by a return to normal daily activity

Ideally all enzymes in the tricarboxylic acid (TCA) cycle and the respiratory chain shod be studied However according to Pette and colleagues the enzyme activities within ea metabolic pathway are closely related (Bass *et al* 1969) If this holds true for humans, i is possible to limit the study to one marker enzyme representing each system. Succinat dehydrogenase (SDH) was selected as a marker for the TCA cycle and cytochrome oxidas for the respiratory chain. Data on changes in skeletal muscle capillarization and morpholog will be reported separately

Materials and Methods

13 healthy men (age 20–23 years, weight 57 110 kg, and height 160–190 cm) participated in the stud They were informed bout the procedure and the risks involved in the experiments before they ohnteed to participate. All subjects perf rmed military service during the time of the study their jobs consisting of office wo k or car-driving. None had been engaged in any regular physical trai ing during the preceding year

The subjects were divided into 2 groups. In th first group (group A) eight subjects were followed during a 10 week period of training (2 of these subjects continued training for an additional 4 weeks), followed by at least 6 weeks of normal physical activity t the pre-training level. An additional group of 5 subjects (group B) was f llowed during an 8 week training period with weekly tests withi the first 3 eeks.

In group A the subjects trained by pedalling a bicycle ergometer 20 min a day an average of 4 times a week Work loads were initially chosen as the maximal load that the subjects could tolerate for 20 min Work-loads were increased as tolerated over the first 2 weeks but were kept constant thereafter A erage load after 2 weeks of training corresponded t 89% $\dot{V}_{O_2}$ max (range 78–98%). Due to the training-induced increase in V_{O_2} max the corresponding value t the end of the training period was 75 (range 64–84).

In group B the training stimulus was progressively increased by continuously adjusting the bicycle work-load after measuring V_{O_2} max so as t keep the relative work-load constant at 80 of the V_{O_2} max The subjects *in this group were stimulated to wo k long as possible d ring each tra ning session* by a monetary reward system. On the average these subjects trained on the bicycle 40 mi a day 4 times per week. The training i both groups was supervised daily

Muscle biopsy sampli g together with V_{O_2} max determinations were performed before as ell as on repeated occasions during the training and de-training periods (Fig. 1 nd 2) On testing day the subjects always came to the laboratory i the morn g after a l ght breakfast. W th few exceptions ll tests and sampling were performed about 7? h after the last traini g session.

Maximal ygen uptak determinations

Pre-training: The following criteria were used to ascertain that V_{O_2} max was achieved respiratory R value bove 1 maximal heart rate and a lower measured o ygen uptake than that predicted f the ork load. In group B at least 3 different V_{O_2} ma determination were made on each subject, using work-loads demanding an oxygen uptak slightly under t nd sl ghtly bove the measured V_{O_2} max.

Training and de-training period The subject al y started to w rk t the same load that h d el ited V_{O_2} max in the preceding test. If the subject was then estimated t be ble t continue f more than 5 min at this load, the work-load was increased until the new V_{O_2} ma was reached. I this way reliable data on the changes in V_{O_2} max during the course of the study were obtained.

Maximal oxygen uptak determinations were perf rmed on bicycle ergometer t 60 rpm. E pired air

TRAINING DE TRAINING

Fig. 1. Changes in $\dot{V}O_2$ max and vastus lateralis oxidative enzyme activities during training and de-training periods. Asterisks denote significant difference ($p < 0.05$) from pre-training (training period) or end of training (the de-training period).

was collected in Douglas bags and subsequently analyzed using the Haldane technique (group A) or Beckman LB-1 infrared CO_2-analyzer and Servomex paramagnetic O_2 analyzer (OA 184) (group B). The accuracy of the latter analyses was verified with the Scholander microtechnique.

Muscle biopsies

Before the $\dot{V}O_2$ max determinations a muscle biopsy (20–40 mg) was taken from the lateral aspect of the quadriceps femoris muscle, 12 to 16 cm above the patella, using the needle biopsy technique (Bergström

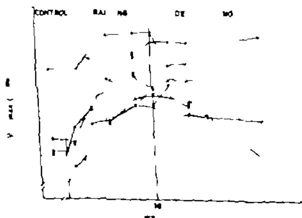


Fig. 2. Individual changes in $\dot{V}O_2$ max during training and de-training periods. Solid lines group A, dashed lines group B.

occurring in a nerve to a slow muscle, growth of new capillaries appears after 4 days a precedes the changes in the activity of oxidative enzymes by 10 days. This informatio suggests that enhanced blood supply and thus oxygen delivery to the muscle may be requir for a stimulation of the synthesis of oxidative enzymes.

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I group B the training stimulus was progressively increased by continuously adjusti g the bicycle work-load after measuring V_{O_2} ma so as t keep the relative work-load constant t 80 of the V_{O_2} max The subjects in this group were stimulated t work as long as possible during each traini g session by monetary rew rd system. On the erage these subjects trained on the bicycle 40 min a day 4 times per week. The training in both groups was supervised daily

Muscle biopsy sampling together with V_{O_2} ma determinations were performed before ell as on repeated occasions during the training and de-training periods (Fig. 1 nd 2). On testing day the subjects always came to the laboratory in the morning after light breakfast. With few exceptions all tests and sampling were performed bout 72 h after the last training session.

Maximal oxygen uptak determinations

Pre-training: The following criteria were used to ascerta that V_{O_2} ma was achieved respiratory R value abo e 1 maximal heart rate and lower measured ygen uptak than that predicted f the ork load. In group B at least 3 different V_{O_2} max determinations were made on each subject, using work-loads demanding an oxygen uptake slightly under t nd slightly bove the measured V_{O_2} max.

Training and de-training periods: The subject alw y started to work at the same load that had lcited V_{O_2} max in the preceding test. If the subject was then estimated to be able to continue for more than 5 min at this load, the work-load was increased until the new V_{O_2} max wa reached. I this w y reliable data on the changes in V_{O_2} max during the course of the study were obtained.

Maximal oxygen uptak determinations were performed on bicycle ergometer at 60 rpm. E pired air

e-training level, but 3 subjects still had a higher $\dot{V}_{O_2}$ max than before the training period (Fig. 2). (One subject could not be studied at this time.) The post-training decline in vastus lateralis oxidative enzyme activities revealed different time-course. 4 weeks post-training the average SDH activity was significantly lower than at the end of training ($p<0.05$) and after further 2 weeks it had declined to the pre-training level. The cytochrome oxidase activity reached this level 2 weeks post-training, and in the following month it tended to decline even further. The de-training decline of vastus lateralis cytochrome oxidase activity expressed in percent of the value at the end of training, was significantly greater than that of $\dot{V}_{O_2}$ max, regardless of whether it was calculated at 2 ($p<0.001$), at 4 ($p<0.001$) or at 6 ($p<0.001$) weeks of de-training. A difference between the decline of $\dot{V}_{O_2}$ max and vastus lateralis SDH activity was found 4 ($p<0.05$) and 6 ($p<0.001$) weeks post-training. The decline of cytochrome oxidase activity as found 2 and 4 weeks post-training was significantly greater than that of SDH ($p<0.005$).

Discussion

During the training period no significant difference between the time-courses of change in maximal oxygen uptake and skeletal muscle oxidative enzyme activities was detected. The latter were assayed under optimal *in vitro* conditions and may be regarded as relative measures of metabolic capacities (Pette 1971). One possible interpretation of the results from the training part of the study is that the body's maximal capacity to utilize oxygen increases as a result of an enhanced oxidative potential in skeletal muscle. However the results from the de-conditioning phase show that an enhanced oxidative potential of skeletal muscle is not a necessity for an increase in total body $\dot{V}_{O_2}$ max. Therefore, the most likely conclusion to be drawn from the present data is that exercise acts as stimulus both for factors underlying an increased $\dot{V}_{O_2}$ max, as well as for oxidative enzyme synthesis in skeletal muscle. Thus adaptation in $\dot{V}_{O_2}$ max is associated with but not dependent on an adaptation in skeletal muscle oxidative capacity. Accordingly discrepancies between increases in muscle oxidative capacity and $\dot{V}_{O_2}$ max have been reported (Gollnick *et al.* 1973, Henriksson and Reitman 1976). Increased muscle oxidative capacity may be more important during submaximal work, contributing to a shift towards greater fat relative to carbohydrate metabolism (Holloszy 1967, Karlsson *et al.* 1974).

The metabolic capacities of the TCA cycle and of the respiratory chain were increased to the same extent during the training period, which confirms earlier reports based on animal studies (Holloszy 1975). The de-training decline in activity of the respiratory chain marker cytochrome oxidase was significantly faster than that of the TCA cycle marker SDH. This is somewhat surprising in view of the fact that both enzymes are situated on the inner mitochondrial membrane and therefore might be expected to turn over with similar half lives (Rabinowitz and Zak 1975). If an exponential curve is fitted for the decline in enzyme activity the half life of the enzyme can be calculated using a first-order kinetic model as described by Schimke and Doyle (1970). Determined in this way the half-life of cytochrome oxidase is 5 days. In view of the limited number of muscle samples analyzed in the de-training period this value is to be considered as an estimate. However it is of the same order of magnitude as reported for cytochrome C turnover in rat muscle, where the calculated

1962). In group B two samples were obtained before the training period, one before and the other after the 3–4 days of V_{O_2} max determinations. As these two samples did not differ significantly in either SDH or cytochrome oxidase activities, the averages were used as pre-training values for group B. Up to seven biopsies were taken from the same subject in group A, alternating between the left and the right leg. In group B all biopsies were taken from the same leg.

The muscle samples were immediately frozen in liquid nitrogen and stored at −80°C for subsequent enzyme analyses. The enzyme analyses were performed within 2–3 months.

For SDH analysis the muscle was homogenized in 0.3 M phosphate buffer (pH 7.7) with 0.05% BSA and analyzed as described previously (Essén et al. 1975).

For cytochrome oxidase analysis the muscle was homogenized in 0.01 M phosphate buffer (pH 7.4) with 0.05% BSA. From the homogenate 100 μl was added to 1 ml of aerated 0.05 M phosphate buffer (pH 7.5). The reaction mixture which was kept at 30°C, was then made 30 mM with respect to N,N,N',N'-tetramethylphenylenediamine-dihydrochloride (TMPD), 50 mM with respect to sodium ascorbate and 10 mM with respect to cytochrome C. The O_2 consumption was then followed with a Clark oxygen electrode. After subtracting the oxygen consumption of a blank solution, where the muscle homogenate had been replaced by the same volume of homogenizing medium, the cytochrome oxidase activity could be calculated.

In six subjects tests were made to determine whether enzyme activities were different in the 2 legs. No such differences were found for either SDH or cytochrome oxidase activities. SD of the difference between the right and the left leg was 1.5 (SDH) and 0.9 μmol · g^{-1} (wet weight) · min^{-1} (cytochrome oxidase), i.e. in both cases, 14% of the mean value. This variability was of the same order of magnitude as that seen when a biopsy was taken from the same vastus lateralis on different days. SD of the difference was then 13% (SDH, n = 18) and 14% (cytochrome oxidase, n = 8) of the mean value.

Statistics. The significance of intraindividual differences was tested using Student's t-test.

Results

In spite of the different design of the training programmes in the 2 groups, no significant differences in changes of either V_{O_2} max or enzyme activities were seen at any time. Thus in the following, the 2 groups will be considered together. All results are presented as per cent change from the pre-training level, which for V_{O_2} max was 3.27 l · min^{-1}, for SDH and cytochrome oxidase activities 9.5 and 7.1 μmol · g^{-1} (wet weight) · min^{-1}, respectively.

During the training V_{O_2} max and vastus lateralis oxidative enzyme activities increased gradually and were significantly different from the pre-training levels after 3 weeks (Fig. 1). *At this time the average V_{O_2} max was 11.1% above the pre-training level* ($p < 0.001$). Vastus lateralis SDH and cytochrome oxidase activities were then 11.5% ($p < 0.05$) and 11.0% ($p < 0.05$) above the pre-training levels, respectively. After 5 and 8 weeks of training V_{O_2} max was 12.6% ($p < 0.001$) and 18.6% ($p < 0.001$) higher than the pre-training level. Corresponding values for SDH and cytochrome oxidase activities were, after 5 weeks, 20.5% ($p < 0.005$) and 25.6% ($p < 0.05$), and after 8 weeks, 32.0% ($p < 0.001$) and 34.7% ($p < 0.005$), respectively. Both after 5 and 8 weeks of training the average increase in muscle oxidative enzyme activities was considerably greater than that of V_{O_2} max, however, the differences were at neither occasion statistically significant.

De-training period. Of the 8 subjects (group A) who were studied in this period 6 discontinued training after 10 and two after 14 weeks. The results at these intervals are indicated as end of training values in Fig. 1.

The average V_{O_2} max at 6 weeks post-training was still 16% higher than the pre-training value ($p < 0.01$) and not significantly different from the value at end of the training ($p > 0.2$). 12 weeks after cessation of training V_{O_2} max in 4 out of the 8 subjects had returned to the

training level, but 3 subjects still had a higher V_{O_2} max than before the training period . 2). (One subject could not be studied at this time.) The post-training decline in vastus alis oxidative enzyme activities revealed a different time-course. 4 weeks post-training average SDH activity was significantly lower than at the end of training ($p < 0.05$) after further 2 weeks it had declined to the pre-training level. The cytochrome oxidase vity reached this level 2 weeks post-training, and in the following month it tended to line even further The de-training decline of astus lateralis cytochrome oxidase activity ressed in percent of the alue at the end of training, was significantly greater than that V_{O_2} max, regardless of whether t was calculated at 2 ($p < 0.001$), at 4 ($p < 0.001$) or at p 0.001) weeks of de-training. A difference between the decline of V_{O_2} max and vastus tralis SDH activity was found 4 ($p < 0.05$) and 6 (p 0.001) weeks post-training. The line of cytochrome oxidase activity as found 2 and 4 weeks post-training was significantly ster than that of SDH (p 0.005).

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half life was 8 days (Terjung 1975). Due to the scatter around the curve, the same type calculation for SDH activity is more uncertain, but the half-life obtained is at least 2 times longer than that of cytochrome oxidase

If the degradation rate of mitochondrial enzymes is of the magnitude indicated by the half life of cytochrome oxidase, this might explain the relatively low percentage incre in skeletal muscle oxidative capacity normally seen in humans with training program of the same type as in the present study De-training effects between exercise bouts r influence the rise in oxidative enzyme activities during the training period, making it l than it would theoretically be if a maximal training stimulus could have been impo With a gradually increasing stimulus over a sufficient period of time the oxidative capa of human skeletal muscle can increase considerably Gollnick *et al* (1973) for examp observed a doubling in vastus lateralis SDH activity after 5 months of extensive endura training.

No significant differences in the magnitude and time-course of the adaptive chang between the two training groups were found. However between the fifth and the eighth we of training there was a tendency toward a continuous increase in SDH and cytochrom oxidase activities (20 and 17 per cent) in group B whereas in group A these appeared t have levelled off after the fifth training week. These findings are then in accordance wi the results of Benzi *et al* (1975), who found in rats, that in response to a constant traini stimulus the oxidative enzyme activities tended to level off at a certain level, which the changed if a new level of physical activity was imposed on the animals.

The results of the present study indicate, that with changes in the physical activity leve fast adaptive changes occur both at the local muscle and the systemic level in the maxim capacity to utilize oxygen. The discrepancies in post training decline of maximal oxyg uptake and leg muscle oxidative capacity indicate that the latter is not the limiting fact determining the body s maximal capacity to utilize oxygen During the training period th is further substantiated by the tendency towards a greater percentage increase in skelet muscle oxidative enzyme activities as opposed to maximal oxygen uptake

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References

B ss, A D B DICZKA, P EYER, S. HOFER and D PETTE, Metabolic differentiation of distinct muscl types at the level f enzymatic rganization *Europ J Biochem* 1969 *10.* 198 206

BENZI G P P NCERI, M DE BERNARDI, R VILLA E. ARCELLI, L. D ANGE O, E. ARRIGONI nd F B rl Mitochondrial enzymatic daptation f keletal muscle to end rance t a ng *J appl. Ph* l. 1975 *38* 565–569

B OSTRÖM, J Muscle electrolytes man *Scand J clin. Lab Invest* 196.. Suppl 68.

B OWN M M COTTER, O HUDLICKA, M SMITH and G V BOVA, Metabolic changes in long-term stim lated fast muscles. I *Metabolic Adapt ions to Prolonged Exercise* (Proceed ngs of the 2nd Int. S mp of Biochem. of E ercise M ggl gen 1973)

ESSÉN, B., E. J NSSON, J HENRIKSSON, A. W T YLOR and B S TIN Metabolic characteristics of fibr types human skeletal muscle *A ta ph iol scand.* 1975 *95* 153 165

GOLLNICK P D R B. ARMSTRONG, B. SALTIN, C W SAUBERT W L SEMB OWICH and R. E. SHEPHER Effect of training on enzyme activity and f ber composition of huma sk letal muscle *J appl Ph siol* 1973. *34* 107 111

[illegible]ERSSON, J. and J. S. RITTMAN, Quantitative measures of enzyme activities in type I and type II muscle fibres of man after training. *Acta physiol. scand.* 1976. 97 392 397

[illegible]OLLOSZY J O Biochemical adaptation in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J biol. Chem.* 1967 242. 2278–2282.

[illegible]OLLOSZY J. O Adaptation of skeletal muscle to endurance exercise. *Med. Sci. Sports* 1975. 7 155–164.

[illegible]ARLSSON, J., L.-O NORDESJÖ and B. SALTIN, Muscle glycogen utilization during exercise after physical training *Acta physiol. scand.* 1974. 90. 210–217

[illegible]ETTE, D Metabolic differentiation of distinct muscle types at the level of enzymatic organization In. *Muscle Metabolism During Exercise* (eds. B. Pernow and B. Saltin), Plenum Press, New York–London, 1971 vol 11. 33–49

[illegible]ABINOWITZ, M. and R. ZAK, Mitochondria and cardiac hypertrophy *Circulat. Res.* 1975. 36. 367 374.

[illegible]CHIMKE, R. T and D. DOYLE, Control of enzyme levels in animal tissues. *Ann. Rev. Biochem.* 1970. 39 929–976

[illegible]ERLING, R. L., Cytochrome C turnover in skeletal muscle. *Biochem. biophys. Res. Commun.* 1975 66. 173–178.

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No significant differences in the magnitude and time-course of the adaptive changes between the two training groups were found. However between the fifth and the eighth week of training there was a tendency toward a continuous increase in SDH and cytochrome oxidase activities (20 and 17 per cent) in group B whereas in group A these appeared to have levelled off after the fifth training week These findings are then in accordance with the results of Benzi *et al* (1975) who found in rats, that in response to a constant training stimulus the oxidative enzyme activities tended to level off at a certain level, which then changed if a new level of physical activity was imposed on the animals.

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References

BASS, A D BRDICZKA, P EYER, S. HOFER and D PETTE, Metabolic differentiation of distinct muscle types at the level of enzymatic organization. *Europ J Biochem.* 1969 *10* 198 206.

BENZI G P P NCERI M DE BERNARDI, R. VILLA, E. ARCELLI, L. D ANGELO, E. A [illegible] and [illegible] Mitochondrial enzymatic adaptation of skeletal muscle to endurance training *J appl Physiol.* 1975 *38* 565–569

BERGSTRÖM, J Muscle electrolytes in man *Scand. J Clin. Lab Invest* 196_ *8* ppl. 68.

BROWN, M M COTTER, O HUDLICKA, M SMITH and G VRBOVA, Metabolic changes in long-term stimulated fast muscles. In *Metabolic Adaptations to Prolonged Exercise* (Proceedings of the 2nd Int. Symp of Biochem. of Exercise, Magglingen 1973).

ESSÉN, B E. JANSSON, J HENRIKSSON, A W TAYLOR and B SALTIN Metabolic characteristics of fibre types in human skeletal muscle *Acta ph siol. scand.* 1975 *95* 153–165

GOLLNICK, P D R B. ARMSTRONG, B SALTIN C. W SAUBERT W L. SEMBROWICH and R. E. SHEPHERD Effect of training on enzyme activity and fiber composition of human skeletal muscle. *J appl. Physiol* 1973 *34* 107 111

…SSON, J. and J. S. REITMAN, Quantitative measures of enzyme activities in type I and type II muscle fibres of man after training. *Acta physiol. scand.* 1976, 97, 392–397.

…LLOSZY, J. O. Biochemical adaptation in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J. biol. Chem.* 1967, 242, 2278–2282.

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…BINOWITZ, M. and R. ZAK, Mitochondria and cardiac hypertrophy. *Circulat. Res.* 1975, 36, 367–374.

…HIMKE, R. T. and D. DOYLE, Control of enzyme levels in animal tissues. *Ann. Rev. Biochem.* 1970, 39, 929–976.

…RJUNG, R. L. Cytochrome C turnover in skeletal muscle. *Biochem. biophys. Res. Commun.* 1975, 66, 173–178.

Acta physiol. scand. 1977 99 98–104
From the Physiological Section, Institute of Medical Biology University of Tromsø, Norw

The Effect of Plasma Proteins on the Capillary Permeability in the *rete mirabile* of the Eel (*Anguilla vulgaris L.*)

By

KJELL MYHRE and JOHAN B STEEN

Received 14 July 1976

Abstract

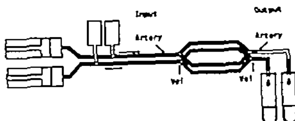
MYHRE, KJ and J B STEEN *The effect of plasma proteins on the capillary permeabil the* rete mirabile *of the eel* (Anguilla vulgaris L.) Acta physiol scand 1977 99 98-1

Measurements of transcapillary exchange of high-molecular dextrans, K and THO in the *rete mir* of the eel (*Anguilla vulgaris* L.) have been made under steady-state cond tions while varying the amo of plasma proteins in the perfusates. When the perfusates contained 20% or less horse serum the per abilities of D-75 000, K and THO increased significantly The effect on the K permeability as irre ible while that on D-75 000 and THO was not. The permeability was unaffected when the perfusate c tained 30% serum or more. Adding 1 3 g/100 ml bovine albumin to the perfusat maintained the D-75 and THO permeabilities unchanged while that of K^+ appeared to increase. At an albumin content of g/100 ml the effect was similar to that produced by low serum perfusates. Our main conclusion is that of the albumin fraction f the plasma proteins reversibly increases the ntercellular pore area and irrever changes the K^+ permeability of the cell membrane.

Krogh and Harrop (1921) demonstrated that when the leg of the common frog (*R temporaria*) was perfused with a Ringer solution containing 3% gum-acacia, the capillar dilated and the tissue became edematous unless defibrinated ox blood was added to perfusate. Since then several blood constituents have been shown to participate in the ma tenance of normal capillary permeability in the frog. According to Drinker (1927) the dition of 15% to 20% horse serum prevented edema. Saslow (1938) however claimed t red cells had to be present, while Danielli (1940) showed that both red cells and platel retarded the development of edema Recently bovine albumin has been shown to redu the mesenteric capillary permeability of T 1824 in the frog (Levick and Michel 1973 a b

Danielli (1940) suggested that the platelets blocked the protein-permeable pores, wh Landis and Pappenheimer (1963) proposed that the plasma proteins combined loosely w the walls of the capillary pores and reduced their cross-sectional area The latter view b been supported by Levick and Michel (1973 b).

As previously shown by Stray-Pedersen and Steen (1975) and Myhre, Steen and Stra Pedersen (1976) the *rete mirabile* in the swimbladder of the common eel is useful in study capillary permeability It consists of parallel arterial and venous capillaries running clo together as first described by Krogh (1929)



...hematic drawing of the experimental design. The perfusates were delivered to the *rete* from 2 ...n the input artery and vein. The syringes (A and V) are located in perfusion pump and the ...yringe (A) contained the test substances. The input pressures were measured by two pressure ...rs (P) connected to the input tubes by T-couplings. The outflow from the *rete* was collected from ...t artery and vein in two pre-weighed test tubes.

...present expts. the *rete* of the eel was perfused with a Ringer Dextran solution while ...ct on the permeability of Dextran-75 000, K and THO was evaluated at varying ...rations of protein in the perfusates.

Material and methods

... Eels, weighing 200 g to 400 g, were caught in sea water and kept in running fresh water in the ...ory.

...*ry and perfusion.* After making the fish unconscious by a blow on the head the swimbladder was ...d and mounted on a table top. The artery and vein on the heart side of the *rete* (in the present study ...input artery and vein, Fig. 1) were cannulated with polyethylene tubes (Intramedic, R, PE 60 and ...) while small glass tubes were inserted in an artery/vein pair on the bladder side of the rete (termed ...artery and vein). Broad-plated bulldog clamps were used to close the numerous vessels branching ...n the output artery and vein.

...*rete* was perfused in parallel fashion. The input tubes were connected to two glass-syringes driven ...ion Perfusor Pump (Braun-Melsungen) accuracy ±2 % of nominal flow. The perfusate from the ...s collected in pre-weighed test tubes. The rate of output flow was calculated from the weight of the ...and the time of collecting. At the start of the expts. the input flow (0.2 ml/min–0.5 ml/min) was ...d to give perfusion pressures of 30–50 cm H_2O in the input artery and 10–15 cm H_2O in the input ...expose the *rete* to near normal conditions (Mott 1950), and maintained constant throughout the ... The input arterio-venous pressure difference was not allowed to change more than ±2 cm H_2O ...t discarding the expt. The input pressures were measured by two Statham P23 Db transducers con... to the input tubes by side branches (Fig. 1), and continuously registered on a Beckman Dynograph ...R. Further details are described in Myhre, Steen and Stray-Pedersen (1976).

...*fusates.* Horse serum was obtained commercially (Gibco Bio-Cult, Glasgow, Scotland) and main... frozen. Prior to the expts. the serum was thawed in room temperature and filtered.

... Ringer Dextran solution contained 130 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$ 2 H_2O, 5.6 mM ... and 0.43 mM Dextran T 70 (MW 70 000). All chemicals were of analytical grade. The osmolality ... solution was about 290 m Osmol/kg. It matched approximately eel plasma concerning the mono... ions, while the Ca^{2+} content was doubled, and made possible 4 to 5 h perfusion of the rete in vitro ...r nearly stable conditions.

...some experiments bovine albumin (Armour Pharmaceutical Company, Chicago 11, U.S.A.) was ...d to the Ringer-Dextran solution in concentrations 1.3 g/100 ml or 0.33 g/100 ml. This corresponds ...% and 10 % respectively of that present in horse plasma.

...eparinized and frozen horse plasma (20 I.U. Heparin A L/ml) was prior to an expt. thawed at room ...perature and filtered.

...ll perfusates were equilibrated with air prior to the expts. Papaverine sulphate (0.2 mg/ml) was added ...he perfusates in order to secure stable perfusion conditions (Stray-Pedersen and Steen 1975). This solu... crystallized, however, at pH above 6.0. Consequently the pH of all perfusates was adjusted to 5.9 ...h NaOH or HCl.

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Table I. Average permeabilities of D-75 000, K and THO, standard errors of the mean and range of transcapillary input pressures in the initial phase of the experiments.

Number of expts	Range of transcapillary input pressures (cmH_2O)	Average permeability area (PS) ± S.E. (10^{-4} cm^3 s^{-1})		
		D-75 000	K	THO
7	10–48	2.28 ± 0.26	3.97 ± 0.51	97.89 ± 15.22

Results

Table I the permeabilities of D-75 000, K and THO in the initial phase are listed as the mean value of all expts. THO penetrated the capillary walls about 24 times as fast as K which in turn penetrated about 2 times faster than D-75 000.

Fig. 2 illustrates the effect of serum on the vascular bed. The capillary permeabilities of the test molecules obtained with perfusates containing 50% (Group A) and 30% (Group B) horse serum did not differ from those obtained by 100% serum. With lower serum content in the perfusates (Group C–F) the permeability of D-75 000, K and THO increased. Perfusates consisting of 100% Ringer-Dextran (Group F) did not give a significantly different response from that obtained when the rete was perfused with 80% Ringer Dextran and

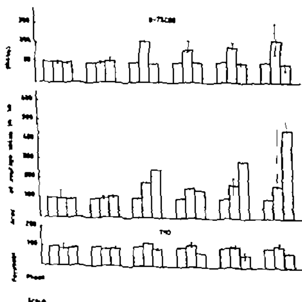


Fig. 2. The effect on the permeabilities of D-75 000, K and THO of different concentrations of serum in the perfusates. In the initial (I) and recovery (R) phase the rete was perfused with 100% serum while the serum in the experimental phase (E) was diluted with Ringer Dextran. The experiments were separated in groups according to the content of serum in the perfusates. (Group A, 50% serum, Group B, 30% serum, Group C, 20% serum, Group D 10% serum, Group E, 1% serum; Group F 0% serum.) The permeabilities for D-75 000 and THO in the experimental phase are averaged from all samples collected, but for K from the last 3 samples only. The permeabilities are expressed in per cent of the average permeability in the initial phase and plotted as the median of N expts. The vertical bars mark the range of the permeabilities in different expts.

Transcapillary gradients were obtained by adding K^+ (as KCl), tritium labelled water THO (Kjeller Norway) and Dextran-Carboxyl-^{14}C (D-75 000), MW 60 000–90 000 (NEC 211 B, New England Nuclear Mass. U.S.A.) to the arterial perfusates. Equimolar amounts of NaCl were added to the venous perfusate to maintain isosmotic conditions.

Experiments. 3 series of expts. were carried out. In the first series we compared the capillary permeabilities of the test substances during serum perfusion to the permeabilities obtained when serum was diluted with Ringer-Dextran. The perfusates contained 100%, 50%, 30%, 20%, 10%, 1 and 0 % horse serum.

Since perfusates containing less than 20% serum caused typical vascular changes, we tested in the second series the effect of bovine albumin. To this end we compared the permeabilities during serum perfusion with those during perfusion with Ringer Dextran containing albumin. Two concentrations of bovine albumin were used. The first corresponded to the content of albumin in a perfusate of 40% horse serum in Ringer-Dextran (1.3 g/100 ml), the second concentration to 10 % serum in Ringer-Dextran (0.33 g/100 ml).

To test for possible effects of substances removed by defibrination of plasma, we compared, in the third series, the permeabilities during serum perfusion with those obtained during horse plasma perfusion.

Each expt. was designed in a standard way. It started with an initial phase of serum perfusion. When stable permeability values had been obtained, the rete was, in the experimental phase, perfused with the test perfusate. The expt. was terminated with a recovery phase of serum perfusion.

The initial phase lasted 30–60 min and 3–4 samples of the outflow were collected. The experimental phase lasted 25–100 min and at least 3 samples were collected. The recovery phase was terminated when the transcapillary input pressures changed more than $\pm$ cm H_2O and as many samples as possible were collected.

The experiments were terminated by perfusing the *rete* with a filtered solution of Indian ink to determine the location of leakages. Leakages in the input vessels or in the *rete* would reduce the flow through the capillaries and when this occurred the expt. was discarded.

Analysis. The concentrations of the test substances were measured in the input and output perfusates. Samples of the input perfusates were collected at the beginning and end of each phase and analyzed in triplicate, while the output samples were analyzed in duplicate.

K: The concentrations of K were measured with a flame photometer (Flame Photometer 343, Instrumentation Laboratory (U.K.) Ltd.). Accuracy was within ± 2 % in the actual range of concentrations (3–9 mEq/l).

THO Dextran-Carboxyl-^{14}C: The Packard Tri-Carb Liquid Scintillation Spectrometer Model 3375 was used to measure the concentrations of the radioactive labelled test substances. To achieve adequate counting accuracy the activities in the input perfusates were adjusted to obtain at least 10 000 counts in the outflow samples. Instagel (Packard Instrument Co.) was used as scintillation liquid.

Calculations. The permeabilities of the test substances were calculated from steady-state retial flow and concentrations measured in the input and output perfusates. The term permeability as it is used in this study refers to total exchange either carried by bulk flow or by diffusion. Bulk flow will be most important for Dextran, diffusion for K and especially for THO (Myhre, Steen and Stray-Pedersen 1976). The area available for transcapillary exchange in the *rete* was not measured. Hence the permeability (PS) is expressed as the product of the permeability coefficient (P) and the area (S), according to (1)

$$PS = \tfrac{1}{2}\, W \, \frac{\Delta C_a}{\Delta C_L} \quad (1)$$

W = arterial (venous) flow

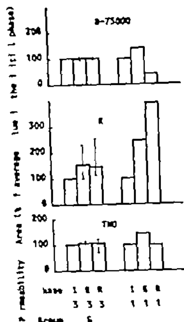
ΔC = arterio-venous concentration difference of the test substances in the input perfusates

ΔC_L = arterio-venous concentration difference of the test substances in the output perfusates

The concentrations of K^+ in the output perfusates will be affected by contamination of intracellular potassium. If we assume that the arterial and venous perfusate receives approximately the same amount of potassium from the tissue, the permeability calculated according to (1) will not be affected. An imbalance will, however, appear between the apparent K gain in the venous perfusate and the K loss from the arterial perfusate.

Both analytical procedures and the transcapillary exchange will influence the accuracy of the permeabilities. Consequently it ranges from about 5% to about 10 % with the highest accuracy for the most permeable substances.

The results were treated statistically with the Wilcoxon Matched-pairs Signed-ranks test (Siegel 1956, p. 75).



g 4 The effect of albumin in the perfusates on the permeabil s of D-75 000, K and THO. In Group G the Ringer Dextran rfusate contained 1.3 g/100 ml bovine albumin, in Group H e albumin content was 0.33 g/100 ml. The permeabilities are pressed as per cent of the average permeability of the initial ... and plotted as the median of N expts. The vertical bars denote the range of the permeability. Abbreviations as in Fig. 1.

When the rete was perfused with horse plasma the capillary permeability did not differ from the values obtained when the perfusate contained 100 % serum.

Discussion

The present study demonstrated that the capillary permeability of the eel *rete* increased when the perfusate contained less than 30 % horse serum, or bovine albumin corresponding to less than 40 % serum. Krogh and Harrop (1921) did similar observations on frog muscles perfused with Ringer solution containing 3 % gum-acacia and supplemented with ox serum. It is therefore suggested that the albumin fraction of plasma takes part in maintaining normal capillary permeability in the rete.

Lack of serum in the perfusate affected the permeability of K differently from that of D-75 000 and THO. Contrary to D-75 000 and THO the K permeability increased progressively during perfusion with low serum perfusates. The effect on D-75 000 and THO must reside in the intercellular pores since it was readily reversed. The effect on K was not reversible and resides most likely in the K -pores of the endothelial cell membranes. This conclusion is supported by the observed leakage of K from the retial tissue during perfusion with solutions containing 20 % or less serum.

Adding 1.3 g/100 ml bovine albumin gave a certain increase of the K permeability. Aside from the fact that the albumin was bovine, this observation might indicate that either higher albumin levels or some other serum fraction was needed to stabilize the cell membranes. In this connection one should note that the experiments of Stray-Pedersen (197 indicated that the protein level which is necessary to maintain normal permeability cond

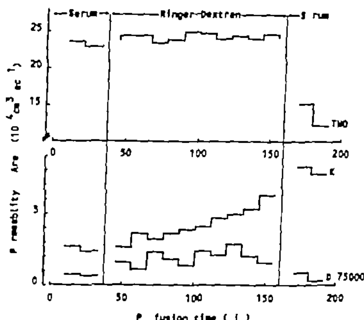


Fig. 3. The effect on the permeabilities (PS) (10^{-4} cm^3 sec^{-1}) of D-75 000, K and THO when the *rete* was perfused with a Ringer-Dextran solution. The transcapillary input pressure remained constant throughout the expt.

20% horse serum. The permeabilities obtained during perfusion with less than 20% horse serum in the perfusate were significantly higher than those obtained during serum perfusion ($p < 0.002$ for Groups C, D, E and $p < 0.005$ for Group F).

During perfusion with less than 30% serum there was clear evidence of leakage of potassium from the retial tissue. This leakage was manifested as an apparently larger gain in venous K^+ concentration than should be expected according to the decline in arterial K concentration. Analysis of cellular ionic changes was not performed.

The increased permeability of D-75 000 and THO produced by low serum concentrations in the perfusate could be restored to normal by perfusing the *rete* with horse serum. In contrast, the increased K^+ permeability could not be reversed. Perfusates with low serum contents also produced other signs of major vascular changes. In several experiments the input pressures started to increase and the *rete* appeared edematous after about 30 min of perfusion with no detectable vascular changes. This is the reason why the number of recovery expts. is reduced in several expts.

The change in permeability of the three test substances progressed differently during perfusions with low serum solutions. The K permeability was increased from the first sample and continued to increase throughout the perfusion period. The permeabilities of D-75 000 and THO, however, increased initially and were maintained at fairly stable levels through the experimental phase. Fig. 3 shows results from a typical expt. in which the perfusate was 100% Ringer Dextran.

Fig. 4 shows the effect of bovine albumin. No change in the permeabilities of D-75 000 and THO was observed when the Ringer Dextran solution contained 1.3 g/100 ml albumin, whereas the albumin concentration of 0.33 g/100 ml produced the same effect as when the Ringer Dextran perfusate contained 20% or less serum. The K permeability increased slightly but statistically not significantly at an albumin concentration of 1.3 g/100 ml, but markedly when the concentration was 0.33 g/100 ml. The K permeability did not return to normal in the recovery phase.

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Skeletal Muscle Fiber Splitting Induced by Weight Lifting Exercise in Cats

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Abstract

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However recent observations indicate that an increase in the number of muscle fibers yperplasia) as well as increase in cross-section area (hypertrophy) of the individual fibers n be induced in muscles trained after removal or denervation of synergistic muscles or ter endurance training (VanLinge 1962, Edgerton 1970, Hall-Craggs 1970, Reitsma 1970, ames 1973 Sola *et al* 1973). The histological picture of these overworked muscles also hows degenerative changes as .g hyaline degeneration, karyopyknosis and monocyte nfiltration. It seems, therefore, of importance to establish experimental evidence whether he reported hyperplasia also occurs in training of unrestrained animals during weight fting exercise. Such an investigation was the aim of the present study

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The observed ability of serum proteins to maintain normal permeability in the rete supports similar observations in the frog (Drinker 1927 Saslow 1938, Danielli 1940, Levick and Michel 1973 a, b Mason, Michel and Tooke 1973). This effect seems not to be related to the osmotic activity of the plasma proteins, since the *rete* vessels were perfused with isosmotic solutions. The increased capillary permeability cannot be produced by inadequate oxygenation of the perfusate, as claimed by Saslow (1938), since all the perfusates in the present study were equilibrated with air

Contrary to Danielli (1940) who proposed that the intercellular pores were specially blocked by platelets, Lardis and Pappenheimer (1963) suggested that the intercellular pore area was normally reduced by adsorbed plasma proteins. Their view was supported by Levick and Michel (1973 a, b) who reported that bovine albumin halved the permeability of T 1824 in the mesenteric capillaries of frogs. The results of the present study represent further support of the view that the albumin fraction of the plasma proteins reduces the cross-sectional area of the intercellular pores.

References

DANIELLI, J F Capillary permeability and oedema in the perfused frog. *J Physiol.* (Lond) 1940. 98 ... 179

DRINKER, C. K. The permeability and diameter of the capillaries in the web of the brown frog (*R. temporaria*) when perfused with solutions containing pituitary extract and horse serum. *J Physiol* (Lond) 1927 *63* 249–269

KROGH, A. *The anatomy and physiology of capillaries.* Yale University Press, 1929 Reprinted 1959 Hafner Publ. Co N Y

KROGH A. and G A. HARROP On the substance responsible for capillary tonus. *J Physiol* (Lond) 1921 *54* 125

LANDIS, E. M and J R. PAPPENHEIMER, Exchange of substances through the capillary walls. In *Handbook of Physiology* Section 2, Circulation. Eds. W F Hamilton and P Dow American Physiological Society Washington D C. 1963. pp. 961 1034

LEVICK J R. and C. C. MICHEL, The permeability of individually perfused frog mesenteric capillaries to T 1824 and T 1824-albumin as evidence for a large pore system. *Quart J exp Physiol* 1973 a 58 67–85

LEVICK, J R. and C. C. MICHEL, The effect of bovine albumin on the permeability of frog mesenteric capillaries. *Quart J exp Physiol.* 1973 b. *58* 87–97

MASON, J C., C. C. MICHEL and J E. TOOKE, The effect of plasma proteins and capillary pressure upon the filtration coefficient of frog mesenteric capillaries *J Physiol.* (Lond) 1973 *229* 15P 16P

MOTT J C., Radiological observations on the cardio-vascular system in *Anguilla anguilla*. *J exp Biol* 1950. *27* 324–333

MYHRE, K. J B. STEEN and S. STRAY-PEDERSEN, The effect of serotonin and transcapillary pressure on the capillary permeability in the *rete mirabile* of the eel (*Anguilla vulgaris* L.). *Acta physiol scand* 1976 *98* 200–208

SASLOW G The relation between the oxygenation of fluids and the occurrence of edema in the perfused frog web. *Amer J Physiol.* 1938. *124* 360–371

SIEGEL, S *Nonparametric statistics for the behavioral sciences* New York McGraw-Hill Book Company Inc. 1956. pp 312.

STRAY PEDERSEN, S. and J B. STEEN, The capillary permeability of the *rete mirabile* of the eel, *Anguilla vulgaris* L. *Acta physiol scand* 1975 *94* 401–422.

STRAY-PEDERSEN, S., The effect of Ca^{++} Mg^{++} and H on the capillary permeability of the *rete mirabile* of the eel, *Anguilla vulgaris* L. *Acta physiol scand.* 1975 *94* 423–441

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References

DANIELLI, J F Capillary permeability and oedema in the perfused frog. *J Physiol.* (Lond) 1940. 9 129

DRINKER, C. K., The permeability and diameter of the capillaries in the web of the brown frog (*R pomaris*) when perfused with solutions containing pituitary extract and horse serum. *J Ph siol* (L 19?7 *63* 49–269

KROGH A. *The anatomy and physiology of capillaries*. Yale University Press, 19?9 Reprinted 1959 H Publ. Co. N Y

KROGH, A and G. A HARROP On the substance responsible for capillary tonus. *J Physiol.* (Lond) *54* 1?5

LANDIS, E. M and J R PAPPENHEIMER, Exchange of substances through the capillary walls. In *Han of Physiology* Section ?, Circulation. Eds. W F H milton and P Dow American Physiological So Washi gton D C. 1963 pp. 961 1034

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MYHRE, K., J B. STEEN and S. STRAY PEDERSEN, The effect of serotonin nd transcapillary pressur the capillary permeability in the *rete mirabile* of the eel (*Anguilla vulgaris* L.). *Acta ph siol scand.* *94* ?00-208

SASLOW G., The relation between the oxygenation of fluids and the occurrence of edema in the perf frog web *Amer J Physiol.* 1938. *124* 360–371

SIEGEL, S., *Nonparametric statistics for the behavioral sciences*. New York McGraw Hill Book Comp Inc. 1956. pp. 312

STRAY PEDERSEN, S. and J B. STEEN, The capillary permeability of the *rete mirabile* of the eel, *Ang vulgaris* L. *Acta physiol scand.* 1975. *94* 401–4?2.

STRAY PEDERSEN, S., The effect of Ca++ Mg and H on the capillary permeability of the *rete mira of the eel, Anguilla vulgaris* L. *Acta ph siol. scand.* 1975 *94* 423–441

Table I Mean values and S.E. for measured and calculated characteristics of the flexor carpi radialis muscle for control and exercised cats.

		Control cats (N = 10)			Exercised cats (N = 5)		
		Left limb	Right limb	% difference	Left limb	Right limb	% difference
[illegible] (d)	$\bar{x}$	1.34	1.32	−1.49	1.34	1.43	6.72*
	S.E.	(0.136)	(0.135)		(0.201)	(0.220)	
[illegible]al [illegible]mber	$\bar{x}$	8 799	9 001	2.30	7 809	9 081	19.3
					[7 701] [8 086] [7 839]	[10 202] [8 856] [8 185]	
[illegible]fibers†	S.E.	(863)	(845)				
SO	$\bar{x}$	37 1	35 6	−1.50	43.6	37 4	−6.20
fibers	S.E.	(1.60)	(1.59)		(4 17)	(1 49)	
FOG	$\bar{x}$	25 2	28.1	2.90	27 8	37 0	9.20
fibers	S.E.	(3.11)	(2.73)		(4 30)	(4 81)	
FG	$\bar{x}$	37 7	36 4	1.30	28 6	25 5	−3.10
fibers	S.E.	(2.78)	(3.59)		(2.14)	(3 44)	

(Controls [illegible] 6, exercised [illegible] 3). *(Found to be significant $p < 0.05$); [3 measurements for exercised cats]

[illegible] right limb of the total fiber count in the control animals, and the differences between [illegible] limbs were not significant. In the trained animals, the exercised FCR showed 37.4 [illegible] SO fibers compared with 43.6% on the nonexercised side. However these differences were not significant, nor did these proportions differ significantly from those of the control animals. In addition, the distribution of FOG and FG fibers showed no significant differences between the left and right muscles of the control and trained animals (Table I). The lack of significant differences in the relative fiber content of the left and right FCR from trained animals could indicate that the absolute increase in total muscle fibers in the exercised FCR is due to a parallel increase in both fast and slow-twitch fibers. However there was a substantial (9.20%), although not significant increase in FOG fibers. The lack of significance is probably due to small sample size (3) with a large variance. This finding is consistent with the observation that all fiber types undergo significant hypertrophy during weight-lifting exercise, however the increase is greater for the FOG fibers (SO, 10.1%, FG 10.8%, FOG 15.1%, Gonyea and Ericson 1976).

The histological picture showed fiber splitting of both fast-twitch and slow-twitch fibers in the trained muscles (Fig. 1). Even by careful examination of the serial sections, which virtually comprised the total length of the FCR, it was not possible to observe fiber splitting in the control animals. A few fibers were also observed to be splitting in the left FCR of the trained animals only. Even though the left limb is not directly exercised, it is used as a supportive brace during training (Gonyea and Ericson 1976). This finding has the effect of making it more difficult to demonstrate significant differences when the total number of muscle fibers was compared between limbs. The data indicate that one possible mechanism for the significant increase in the total number of fibers in the exercised FCR is due to fiber splitting. Whether satellite cells are also involved in this process as has been postulated by several investigators (Schiaffino *et al.* 1972, James 1973) is not known at this time. To

Methods

A control group of 10 adult cats (body wt. 3 70±0.204 kg) and a training group of 5 adult cats (body wt. 3 57±0.164 kg) were established The control animals were confined to individual cages for a period of 4-8 weeks. The *weight-lifting exercise training* was carried out according to Gonyea and Ericson (1976). The cats were conditioned to move a bar a specific distance with their right forelimb to receive a food reward Weights attached to the bar via a pulley were lifted as the bar was moved. During the first week of training, the animals lifted 100 g and at the end of 19-46 weeks of training, they lifted 1 120±1 (mean±S.E.). In this preliminary study the animals were not exercised until heavier weights could no longer be lifted. Therefore the data must be considered as giving only an indication of the degree of hypertrophy that can be expected from cats that are exercised at heavier loads.

On the day of investigation, the *m. flexor carpi radialis* (FCR) was dissected during Nembutal anesthesia and weighed. The cat was then sacrificed with an overdose of Nembutal and the remaining 37 named muscle groups of both anterior limbs were removed and weighed The precautions outlined by Le (1917) in obtaining muscle specimens were adhered to in this study In this regard, the muscles were removed from their attachments by sub-periosteal incision and cleaned of all extraneous connective tissue and fat. All tendon not directly receivi g muscular fibers was removed The muscle weights from the "nonexercised" left limb were then compared to those from the exercised right limb of the trained animals using the t distribution. A probability of less than 0.05 was considered significant The muscle weights of the left and right limbs were also compared for the nonexercised co trol animals.

The *histochemical xamination* was performed on the right and left FCR of 6 control and 3 trained animals. Fresh frozen serial sections (8 μm) were processed for histochemically demonstrable alkali and acid-stable myosin adenosine triphosphatase (ATPase) activity (Guth and Samaha 1970), reduced nicotinamide adenine dinucleotide tetrazolium reductase (NADH TR) activity (Novikoff *et al.* 1961), and for glycogen using the PAS staining technique This muscle was chosen for detailed study because of its small size, and it has been demonstrated to undergo consistent hypertrophy (Gonyea and Ericson 1976). The total number of muscle fibers was counted from a photographic montage taken from a transverse section through comparable regions at the largest girth of the right and left FCR (Sola *et al.* 197 this way the total umber of slow twitch high o idative (SO) and fast-twitch fibers was decided. The distribution of fast-twitch fibers among subgroups, fast-twitch-o idative glycolytic (FOG) or fast-twitch glycolytic (FG) (Peter *et al* 1972), was decided from 6 selected areas of the photo-montage during simultaneously microscopy of the original histochemical sections. The 6 selected reas cont ined a total of 500 fast twitch f bers.

Results and Discussion

The exercise regimen used in this study has already been demonstrated to induce significant muscle hypertrophy (from 7 to 34%) in the exercised limb (Gonyea and Ericson 1976). In this study the control animals showed no significant difference in wet weight comparison between left and right FCR ($p > 0.05$) However in the trained cats, the weight of the exercised right FCR was significantly greater than that of the nonexercised left limb ($p < 0.05$)

The number of muscle fibers and the distribution among slow-twitch and fast twitch fibers in FCR showed no significant difference among right and left sides in the control animals (Table I). In the trained animals, however there was a significant increase, 19. in the total number of fibers of the exercised FCR when compared with that of the nonexercised left FCR ($p < 0.05$). This is a substantial difference when compared with that of the nonexercised cats (2.30%) It can also be observed that the average number of fibers in the right FCR is slightly greater in the exercised animals than that from the control animals even though the average weight of the control animals was greater than that of the exercised animals. Slow-twitch oxidative fibers were 37 1 % in the left 1 and 34 6,

References

…TON, V. R. Morphology and histochemistry of the soleus muscle from normal and exercised rats. …er J Anat 1970, 127 81–88.
…ETON, V. R., R. J. BARNARD, J. B. PETER, C. A. GILLESPIE and D. R. SIMPSON, Overloaded skeletal … nonhuman primate (Galago senegalensis) Exp Neurol 1972, 37 322–339.
… J and G. C. ERICSON, An experimental model for the study of exercise-induced skeletal …pertrophy J appl Physiol 1976 40 630–633.
…nd F. J. SAMAHA, Procedure for the histochemical demonstration of actomyosin ATPase. …rol 1970 28 365–367
…on, E. C. B. The longitudinal division of fibres in overloaded rat skeletal muscle. J Anat …970 107 459–470
… Compensatory hypertrophy in the extensor digitorum longus muscle of the rat. J Anat … 57–65.
… M. Observations on denervated and regenerating muscle. J Physiol (Lond.) 1917 51 377 395.
…, R., Über Aktivität-Hypertrophie der willkürlichen Muskeln. Virchow Arch. path. Anat … 522 554
A. B. W. SETH and J. DRUCKER, Mitochondrial localization of oxidative enzymes. Staining …th two tetrazolium salts J biophys. biochem. Cytol 1961 9 47–61
… Histochemical, biochemical and physiological studies of skeletal muscle and its adaptation … In Contractility of Muscle Cells and Related Processes. Symposium of the Society of General …ists. Prentice-Hall (Englewood Cliffs, New Jersey) 1971 pp. 151–173.
… R. J. BARNARD, V. R. EDGERTON, C. A. GILLESPIE and K. E. STEMPEL, Metabolic profiles of …r types of skeletal muscle in guinea pigs and rabbits. Biochemistry 1972, 11 2627–2633.
… Some structural changes in skeletal muscles of the rat after intensive training. Acta Morphol. …cad 1970 7 229–246.
…, S. S. PETROSOV BORISOVA and M. ALOVE, Cell proliferation in rat skeletal muscle during early … compensatory hypertrophy Virchows Arch. Abt. B Zellpath. 1972, 11 268–273.
… M. D. L. CHRISTENSEN and A. W. MARTIN, Hypertrophy and hyperplasia of adult chicken …latissimus dorsi muscles following stretch with and without denervation Exp Neurol 1973, …00
… B. The response of muscle to strenuous exercise. J Bone Jt. Surg 1962 44 B, 711–721



Fig. 1 Spaced serial sections trained muscle incubated alkali-stable ATPase showing cessive stages (a–f) in the spl of fast-twitch-glycolytic ( ).

the authors knowledge, this is the first time fiber splitting has been demonstrated to oc during a training regimen not inducing degenerative changes of muscle fibers, and dur condition where the unrestrained animal performs weight-lifting exercise during volun contractions.

The present results also show definite muscle hypertrophy due to training. The lack significant muscle hypertrophy during training of unrestrained animals without previ surgical intervention so often reported in the literature (cf Peter 1971 Edgerton et 1972) is most likely due to the endurance type of training (treadmill running, swimmi used in these studies. As shown by Gonyea and Ericson (1976), and in the present stu weight-lifting exercise in the unrestrained animal induces significant muscle hypertrop

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TABLE 1. Weight and choline acetyltransferase activity of parotid glands in untreated rats and in rats treated with pilocarpine for 10 days. For the estimations of the enzyme activity the glands of each rat were pooled. Number of observations is indicated in brackets. Values are mean ± S.E.

	Wet weights mg	Pilocarpine/Control percentage	Enzyme activity in μg ACh/h/pooled glands	Pilocarpine/Control percentage	Enzyme activity in μg ACh/h/g acetone powder	Pilocarpine/Control percentage
Pilocarpine	133.8 ± 5.7 (16)	103.9 ± 3.4	39.3 ± 1.9 (8)	93.0 ± 1.9	740 ± 30 (8)	92.4 ± [illegible]
Untreated	130.3 ± 6.3 (16)		42.2 ± 1.7 (8)		806 ± 44 (8)	

0.1 0.02 0.01 when the glands of the pilocarpine treated rats were compared with those of the untreated control litter mate.

[illegible] collected for 3 1/2-4 h and from the submaxillary gland for 4-5 h in 2 rats examined. The volume of saliva [illegible] was in one of the rats 0.59 ml from the parotid and 1.53 ml from the submaxillary gland, and in the other rat 0.42 ml and 2.28 ml, respectively. A similar relationship between the two glands as to the amount [of saliva] secreted in response to an injection of pilocarpine was found by Holloway and Williams (1965). [In] contrast to larger doses of pilocarpine, the dose of 1 mg did not cause any diarrhoea in the animals.

The rats were killed with ether, the drug treated rats 9-12 h after the last injection of pilocarpine. The parotid glands were then carefully taken out, cleaned, washed in saline, pressed gently between gauze pads and weighed. For the estimation of choline acetyltransferase activity the parotid glands of each animal were pooled. The acetone dried powder of the glands was made up in cysteine-saline in a concentration of [illegible] The activity of choline acetyltransferase was determined according to the method of Hebb (see [also] Ekström 1963, 1965). Of the tissue extract 0.2 ml was incubated for 1 h at 38°C. The acetylcholine formed was estimated on the frog rectus and expressed in μg acetylcholine chloride formed per h per pool of glands [(total activity)] and in μg acetylcholine formed per h per g acetone powder (concentration). The tissue extracts [from] the drug treated rat and the untreated litter mate were incubated simultaneously and the incubates were assayed on the same rectus muscle.

The weights of the glands were expressed both in terms of wet weights and dry weights (acetone dried powder).

For statistical analysis Student's t-test was used, paired comparisons were made between the treated rat and its untreated litter mate. P values less than 0.05 were considered significant.

Results

Neither at the start nor at the end of the experimental period were there any differences in body weights between the drug treated rats and their untreated controls.

As shown in Table 1 the wet weights of the parotid glands were unchanged after the treatment with pilocarpine: this was also the finding when the dry weights were considered. It is also shown in the table that the total enzyme activity decreased ($p < 0.01$) in the parotids of the drug treated rats by about 7 [%] and that the enzyme concentration decreased ($p < 0.02$) by about 8 %.

Discussion

It appears reasonable to attribute the lowered choline acetyltransferase activity found in the parotid glands to a decreased traffic of secretory impulses along the parasympathetic nerve of the glands: the activation of oral and pharyngeal receptors was probably reduced due to the secretion of saliva into the mouth evoked by the action of the parasympathomimetic

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From the Institute of Physiology University of Lund, Sweden

Choline Acetyltransferase Activity in Parotid Glands of Rats after Prolonged Treatment with Pilocarpine

By

JÖRGEN EKSTRÖM

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Abstract

EKSTRÖM, J *Choline acetyltransferase activity in parotid glands of rats after prolonged ... ment with pilocarpine* Acta physiol. scand 1977 99 110–112.

The enzyme activity expressed as total activity and concentration, was found to be decreased in the choler gic neurones of the glands fter treatment with pilocarpine for 10 days. The secretion of saliva evoked b the parasympathomimetic drug is thought to hav reduced the reflex stimulation of the glands b t chollnergic secretory nerves nd thereby lowered the enzyme activity in the nerves.

In parotid glands of the rat the activity of the acetylcholine synthesizing enzyme, chol acetyltransferase has been found to change when the consistency of the food is altered, t activity decreases when the animals are kept on a liquid diet (Ekström 1973), while it i creases when the diet is dry and bulky (Ekström 1974 a). Further procedures causing ness of the mouth such as treatment with an atropine-like drug or salivary duct ligations followed by increased enzyme activity in these glands (Ekström 1974 a, 1975 a). The eq support the concept that the activity of choline acetyltransferase is dependent on the traf of impulses along a nervous pathway (see Ekström 1975 b 1976) when the reflex stimu tion from the mouth is diminished the enzyme activity in the cholinergic neurones of t glands falls, while it rises when the stimulation is augmented

The parasympathomimetic drug pilocarpine is known to cause a profound salivation a in the present study the effect of a prolonged treatment with this drug on the choline acety transferase activity in the parotid glands of the rat was examined

Methods

Female rats, 7 months old and weighing about 200 g, of Sprague Dawley strai bred at thi Institute (used The rats were given a standard pelleted diet and w ter *ad libitum* Pilocarpine hydrochloride, 1 mg 0.2 ml saline, was given s c. every 6 h f 10 days to 8 rats controls served litter mates to the dr treated animals. I control experiments rats were anesthetized with chloralose and fine glass cannulae we inserted into the ducts of the parotid and the submaxillary glands. The dose of pilocarpine used as fou ... flow of saliva within 1 min from both types of glands. The secretion from the parotid gla

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From the Alcohol Research Group of the Swedish Medical Research Council, Institute of Zoophysiology Uppsala, Sweden

Influence of Acetate on the Metabolism of β Hydroxybutyrate in the Perfused Hind-Quarter of the Rat

By

N. Karlsson, E. Fellenius and K.-H. Kiessling

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Abstract

Karlsson, N., E. Fellenius and K.-H. Kiessling. *Influence of acetate on the metabolism of β-hydroxybutyrate in the perfused hind-quarter of the rat* Acta physiol. scand. 1977 99 113–122.

The effect of acetate, at concentrations normally found during ethanol combustion in intact animals and man [illegible] on the uptake and oxidation of β-hydroxybutyrate by the perfused rat hind-quarter was studied. The addition of acetate did not significantly affect the total uptake of β-hydroxybutyrate, but caused [illegible] 40 decrease in the oxidation of β-hydroxybutyrate to CO_2. The oxidation of ^{14}C-β-hydroxybutyrate to $^{14}CO_2$ accounted for about 10 of the total oxygen consumption by the perfused muscle in the absence of acetate. In the presence of acetate this figure was reduced to about 5 %. The addition of β-hydroxybutyrate did not significantly affect the metabolic fate of ^{14}C-acetate. It is concluded that acetate is preferred as oxidative substrate to β-hydroxybutyrate. The inhibited oxidation of β-hydroxybutyrate by acetate did not affect the concentration ratio between β-hydroxybutyrate and acetoacetate in the medium at the end of the perfusion, indicating that the ability of the muscle tissue to restore an increased redoxlevel, "exported" from the liver during ethanol combustion to extrahepatic tissues, was not impaired by acetate.

Key words: Acetate metabolism, ketone body metabolism, skeletal muscle, muscle perfusion, ethanol consumption

The interaction between ethanol and ketone-body metabolism in intact animals and man is still an unsettled problem. In the liver the major site of ketone-body production (Williamson and Hems 1970), ethanol inhibits ketone-body production by diverting free fatty acids to triglyceride synthesis (Fellenius *et al.* 1973 a) and by direct inhibition of the β-oxidation step (Williamson *et al.* 1969 Fellenius and Kiessling 1973). In addition, ethanol combustion may strongly impair the oxidation of acetyl-CoA in the citric acid cycle (Majchrowicz 1975).

The combustion of ketone-bodies is known to be of significant importance for the energy-metabolism of the resting skeletal muscle (Ruderman *et al* 1971 Williamson and Hems

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drug. The decrease in the total enzyme activity by about 7% was less than that found in t parotid glands of rats maintained on a liquid diet for 4–18 days, where the activity was r duced by 22–28% (Ekström 1973). The relatively small decrease observed might be e plained by the fact that the pilocarpine treatment chosen was not sufficient to cause a co tinuous secretion throughout the 24 h, as judged from the results of the control exps. i which the salivary ducts were cannulated and the secretion of saliva studied after a sin injection of pilocarpine. Larger doses or shorter intervals between the injections than the used were considered unsuitable not only because side effects such as diarrhoea would h appeared but also because a stimulating action of the drug on the autonomic ganglia co then be expected: both the amylase rich secretion and the weight increase of the paroti found after administration of big doses of pilocarpine to rats are known to be β-adrenerg effects mediated via the superior cervical ganglion (Schneyer 1965, 1969, Schneyer and H 1965). There was no glandular enlargement in the present study: Schneyer (1969) injecte 10 mg per day (divided into 3 doses) for 10 days also and found an increase in weight 22%. Obviously it is difficult to exclude the possibility of some action of the drug at the ganglionic level. If such an effect were to be exerted on the parasympathetic ganglion th could mean that nervous impulses were set up, thereby influencing the choline acetyltrans ferase activity and counteracting the effect of pilocarpine aimed at. When considering the effect on the sympathetic ganglion, it should be mentioned that stimulation of the β-adrene ceptors of the parotids with isoprenaline over a long time period is not followed by an changes in the total choline acetyltransferase activity (Ekström 1974 b).

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References

Ekström, J. Choline acetyltransferase and secretory responses of the rat's salivary glands after liquid die *Quart. J. exp. Physiol.* 1973. *58.* 171–179.

Ekström, J. Choline acetyltransferase activity in rat salivary glands after cellulose rich diet or treatm with an atropine-like drug. *Quart. J. exp. Physiol.* 1974 a. *59.* 191–199.

Ekström, J. Choline acetyltransferase activity in rat salivary glands enlarged by isoprenaline treatmen *Acta physiol. scand.* 1974 b. *92.* 272–275.

Ekström, J. Compensatory increase i choline acetyltransferase activity in salivary glands and diaphrag muscle of the rat. *Acta physiol. scand.* 1975 a. *93.* 525–530.

Ekström, J. *Choline Acetyltransferase i Salivary Glands and some other Organs, and it Dependence Nervous Activity.* Thesis. Rahms Boktryckeri, Lund. 1975 b. 1–49.

Ekström, J. The effect of teeth amputations on the choline acetyltransferase activity of rat submandib glands. *Acta physiol. scand.* 1977. *99.* 48–52.

Holloway, P. J. and R. A. D. Williams, A study of the oral secretions of rats stimulated by pilocarpi *Arch. oral Biol.* 1965. *10.* 237–244.

Nordenfelt, I., Choline acetylase in normal and denervated salivary glands. *Quart. J. exp. Physiol.* 19 *48.* 67–79.

Nordenfelt, I. *Acetylcholine Synthesis in Sali ary Glands.* Thesis. Håkan Ohlssons Boktryckeri, Lund 1965. 1–25.

Schneyer, C. A., Modification of the action of pilocarpine by drenergic blocking gents. *Proc. Soc. ex Biol.* (N.Y.) 1965. *120.* 230–232.

Schneyer, C. A., β-adrenergic effects by tonomic gents on mitosis nd hypertrophy in rat parotid *Proc. Soc. exp. Biol.* (N.Y.). 1969. *131.* 71–75.

Schneyer, C. A. and H. D. H ll, A tonomic pathways involved in sympathetic-lik action of pilocarpin on salivary composition. *Proc. Soc. xp. Biol.* (N.Y.). 1966. *121.* 96–100.

TABLE I. Rates of β-hydroxybutyrate, acetate and oxygen uptake and net production of lactate in the perfused hind-quarter of the rat.

Additions to perfused medium	β-hydroxy-butyrate uptake	Acetate uptake (μmol/h g wet weight)	Oxygen consumption	Net production of lactate
Control[a]	—	—	27.3 ± 1.4 (5)	0.1 ± 0.8 (5)
Acetate, 2 mM infusion	—	8.0 ± 0.5 (7)	30.6 ± 1.8 (6)	2.3[c] ± 0.4 (7)
β-hydroxybutyrate 7.5 mM	0.87 ± 0.13 (8)	—	23.5[b] ± 1.1 (8)	1.4[c] ± 0.3 (6)
Acetate, 2 mM infusion β-hydroxybutyrate 7.5 mM	0.79 ± 0.10 (10)	7.3 ± 0.2 (8)	29.9[b] ± 0.7 (12)	2.1[c] ± 0.4 (9)

Results are expressed as mean ± S.E. with the number of observations in parentheses. Rat hind-quarters were perfused for 70 min. The standard medium contained glucose, 5.5 mM. Substrates were added at 0 min as single doses (D,L-3-hydroxybutyrate) or as single doses, followed by continuous infusion (acetate 8 μmol/h g wet weight of perfused muscle) (Karlsson et al. 1975).

[a] Data taken from Karlsson et al. (1976).

[b] Significantly different from perfusion with acetate without β-hydroxybutyrate, p < 0.01.

[c] Significantly different from perfusion with β-hydroxybutyrate without acetate, p < 0.01.

[c] Significantly different from control, p < 0.05.

...frozen and treated with $HClO_4$ as before (Karlsson et al. 1975). The perchloric acid extract obtained was analyzed for adenosine-triphosphate (ATP) (Lamprecht and Trautschold 1962), adenosine-diphosphate (ADP) and adenosine-monophosphate (AMP) (Adam 1962), creatine-phosphate (CP) (Lamprecht and Stein 1962), inorganic phosphate (P_i) (Bagnara et al. 1970) and lactate and pyruvate (Hohorst et al. 1959).

Radioactivity measurements

The metabolic fates of the added substrates were studied with U-[14]C-acetate and D,L-3-hydroxy(3-[14]C)-butyrate. The labelled substrates were added to give a specific activity of 9 nCi/μmol of substrate in the medium. Added label was recovered at the end of the perfusion as described previously (Karlsson et al. 1975, Karlsson et al. 1976). The recoveries of added radioactivity are given in Tab. II and III. Uptake of label from the medium was calculated from the change in substrate concentration and the specific activity of the added labelled substrates.

Statistical analysis

Students' t-test was used for statistical analysis, p < 0.05 being considered statistically significant.

Results

Uptake of β-hydroxybutyrate, acetate and oxygen and the net production of lactate

The uptake of β-hydroxybutyrate from the medium (Table I) was linear during the experimental period. The net production of acetoacetate did not exceed 5% of the total uptake of β-hydroxybutyrate whether acetate was present or not (control value 0.04 ± 0.04 μmol/h g (8); in the presence of acetate 0.04 ± 0.01 μmol/h g (10)). The addition of acetate did not change the uptake of β-hydroxybutyrate (Table I). Acetate was continuously infused into the perfusion medium to maintain a constant level of acetate during the experimental period, imitating the situation during administration of ethanol to man and animals in vivo (Majchrowicz 1975). The addition of β-hydroxybutyrate caused no significant changes in acetate uptake (Table I).

1970) It has also been shown previously that acetate, the major product of hepatic ethr oxidation (Lundquist 1962) is readily oxidized by skeletal muscle (Karlsson *et al.* 19 The administration of ethanol to animals and man *in vivo* is generally followed by a 15-fold elevation of the concentration of acetate in the peripheral blood (Majchrowicz 19 The extent to which acetate interferes with glucose and palmitate metabolism in the rat skeletal muscle has been outlined recently (Karlsson *et al* 1976 a, b). The present study performed to investigate the interaction between acetate and β-hydroxybutyrate in t perfused hind-quarter of the rat

Material and Methods

Animal treatment

Female Sprague Dawley rats, weighing 200-240 g purchased from Anticimex, Sollentuna, Sweden w used The rats were fed *ad libitum* on a standard small animal diet (Astra Ewos, Sodertälje, Sweden, r had free access to water prior to the experiments.

Reagent

Standard analytical grade laboratory reagents were obtained from E. Merck AG Darmstadt, West German Enzymes a d co-enzymes were obtained from Biochimica Boehri ger Man heim, West German Sigma Chemical Co., St. Louis, Miss., USA. Solutions of hexobarbital (E ipa [illegible] sod um, Bayer AG Leverkusen West Germany) (100 mg/ml) were freshly prepared from hexobarbital powder Bovine se albumin (fraction V) was obtained from Armour Pharmaceuticals Co Ltd Eastbourne, England sol solutions of lbumin (10) were prepared i Krebs-Henseleit high bicarbonate buffer (Krebs and Henseleit 1932) and d alyzed at 4°C gainst fou f ld olume of the same buffer f r 3 24 hr

U C-acetate (59 mCi/mmol) and D L 3-hydroxy(3-^{14}C)butyrate (4 7 mCi/mmol) ere purchased fro the Radiochemical Center Amersham, England. Chemicals for measurement of radioact ity ere obtained from Packard Instr Co Inc., Ill., USA and Koch Light Laboratories Ltd, Buckinghamshire, England

Perfusion technique and xperimental procedure

The method of rat hind-quarter perfusion used was that described by Ruderman *et al.* (1971). All perfusions were done on skinned animals w th the hind legs t rest The perfusion medium w the same as used previously (Karlsso *et al* 1975). The final volume of the medium was 170 ml The initial concentration of lactate i the medium wa about 1 6 mM Glucose consumption and lactate production due to glycolysis in the bovine red cells duri g the experiment l period was negligible

The rats were anaesthetized by intra peritoneal injections of hexobarbital (25 mg, 100 g b.wt.) The first 25 ml of the perfusat was discarded and the med um was then recycled Substrates t he studied were added at 10 min. D L 3-hydroxybutyrat (0.2 M stock solution w ter) wa added as a single dose to give a final concentration of 0.75 mM of the D-isomer in the medium. Acetate (1.5 M stock solution in water) was added as si gle doses, f llowed by continuous infusion of acetat i to the medium t a rate of 7 0 µmol/h g (Karlsson *et al* 1975). Samples of the med um f r alysis of acetate, acetoacetate, β-hydr o ybutyrate lactate nd pyruvate were taken at 15, 40 and 70 min.

Anal tical methods

Samples of the medium for nalysis of the concentrations of acetoacet te, β-hydro ybutyrate, lactate and pyruvate were deproteinized in ice-cold 0.6 M HClO The extracts were neutralized w th KOH nd the precipitated KClO was removed by centrifugation. The concentrations of acetoacetate (Mellanby and Williamson 1970), β-hydro ybutyrate (Will amson and M llanby 1970) and lactate and pyru te (Hohorst *et al.* 1959) were measured i these extracts. Acetate was determined i plasma accord g to the method of Bergmeyer and Möllering (1970) A freshly prepared standard sol tion of acetate (2 mM) in albumin (4) was lways run parallel to the plasma samples when acetate wa determined. Determination of ygen consumption by the perfused tissue wa performed with polarigraphic tech ique a described previously (Karlsson *et al.* 1975). Oxygen measurements ere usually done t 25 35, 50 nd 60 min.

At the end of the perfusion a portion of the thigh muscles w rapidly frozen *in situ* with lam plier tongs cooled i liquid nitrogen (Wollenberger *et al* 1960). The frozen tissue 350 mg) w

TABLE I. Rates of β-hydroxybutyrate, acetate and oxygen uptake and net production of lactate in the perfused hind-quarter of the rat[a]

Additions to standard medium	β-hydroxy-butyrate uptake	Acetate uptake (μmol/h g wet weight)	Oxygen consumption	Net production of lactate
Control[d]	—	—	27.3 ± 1.4 (5)	0.1 ± 0.1 (5)
Acetate, 2 mM infusion	—	8.0 ± 0.5 (7)	30.6 ± 1.8 (6)	2.3[c] ± 0.4 (7)
β-hydroxybutyrate 7.5 mM	0.87 ± 0.13 (8)	—	23.3[e] ± 1.1 (8)	1.4[e] ± 0.3 (6)
Acetate, 2 mM infusion + β-hydroxybutyrate 7.5 mM	0.79 ± 0.10 (10)	7.3 ± 0.2 (9)	29.8[b] ± 0.7 (12)	2.1[c] ± 0.4 (9)

[a] Values are expressed as mean ± S.E. with the number of observations in parentheses. Rat hind-quarters were perfused for 70 min. The standard medium contained glucose, 5.5 mM. Substrates were added as single doses (D,L-3-hydroxybutyrate) or as single doses, followed by continuous infusion (acetate 3 μmol/h g wet weight of perfused muscle) (Karlsson et al. 1975).

[d] Data taken from Karlsson et al. (1976).

[b] Significantly different from perfusion with acetate without β-hydroxybutyrate, p < 0.01.

[e] Significantly different from perfusion with β-hydroxybutyrate without acetate, p < 0.01.

[c] Significantly different from control, p < 0.05.

...frozen and treated with $HClO_4$ as before (Karlsson et al. 1975). The perchloric acid extract obtained was analysed for adenosine-triphosphate (ATP) (Lamprecht and Trautschold 1962), adenosine-diphosphate (ADP) and adenosine-monophosphate (AMP) (Adam 1962), creatine-phosphate (CP) (Lamprecht and Stein 1962), inorganic phosphate (P_i) (Bergmark et al. 1970) and lactate and pyruvate (Hohorst et al. 1959).

Radioactivity measurements

The metabolic fates of the added substrates were studied with U-^{14}C-acetate and D,L-3-hydroxy(3-^{14}C)-butyrate. The labelled substrates were added to give a specific activity of 9 μCi/mmol of substrate in the medium. Added label was recovered at the end of the perfusion as described previously (Karlsson et al. 1975, Karlsson et al. 1976). The recoveries of added radioactivity are given in Tab. II and III. Uptake of label from the medium was calculated from the change in substrate concentration and the specific activity of the added labelled substrates.

Statistical analysis

Students t-test was used for statistical analyses, p < 0.05 being considered statistically significant.

Results

Uptake of β-hydroxybutyrate, acetate and oxygen and the net production of lactate

The uptake of β-hydroxybutyrate from the medium (Table I) was linear during the experimental period. The net production of acetoacetate did not exceed 5% of the total uptake of β-hydroxybutyrate whether acetate was present or not (control value 0.04 ± 0.04 μmol/h g (8); in the presence of acetate 0.04 ± 0.01 μmol/h g (10)). The addition of acetate did not change the uptake of β-hydroxybutyrate (Table I). Acetate was continuously infused into the perfusion medium to maintain a constant level of acetate during the experimental period, imitating the situation during administration of ethanol to man and animals in vivo (Majchrowicz 1975). The addition of β-hydroxybutyrate caused no significant changes in the uptake (Table I).

TABLE II The effect of acetate on the metabolic fate of D L-3-hydroxy(3-¹⁴C)butyrate in the perfused hind-quarter of the rat

Additions to standard medium	β-hydroxy butyrate uptake (μmol/h g wet weight)	Incorporation of label into $^{14}CO_2$ (μmol of β-hydroxybutyrate/h g wet weight)	Lipids	Oxygen consumption due to oxidation of ¹⁴C-β-hydroxybutyrate to $^{14}CO_2$ (%)[1]	Recovery of added label (%)
¹⁴C β-hydroxybutyrate 0.75 mM	0.97±0.16 (6)	0.45 ±0.03 (6)	0.033±0.004 (6)	8.1 ±0.4 (6)	91+
¹⁴C β-hydroxybutyrate 0.75 mM Acetate, 2 mM + infusion	0.80±0.13 (6)	0.27*±0.02 (6)	0.029±0.008 (6)	4.1*±0.4 (6)	94+1

Results are expressed as mean ± S.E. with the number of observations in parentheses. Experimental conditions see Table I and Methods.
[1] Correction has not been made for re-oxidation of NADH by lactate dehydrogenase
* Significantly different from perfusion without acetate, $p < 0.01$

Acetate alone did not significantly affect the oxygen consumption by the perfused tissue (Table I). β-hydroxybutyrate lowered the oxygen consumption, as compared to the situation with acetate, and this lowering was restored to the control value by the addition of acetate.

In the control experiments, when no substrate was added to the standard medium, the lactate concentration showed only small changes during the perfusion. When acetate or β-hydroxybutyrate or both were added to the medium there was a net production of lactate by the perfused tissue (Table I).

Influence of acetate on ¹⁴C β-hydroxybutyrate metabolism

The β-hydroxybutyrate taken up from the medium was readily oxidized in the perfused muscle. Thus, about 50% of the ¹⁴C label taken up was found in $^{14}CO_2$, while only small amounts were found in lipids in the chloroform extract of the muscle tissue (Table II). About 40% of the total uptake of β-hydroxybutyrate was found as water-soluble metabolites in the PCA-extract of the muscle tissue. When acetate was added the oxidation of ¹⁴C-β-hydroxybutyrate was significantly decreased (Table II). However, in relation to the total uptake of β-hydroxybutyrate from the medium, there was no statistically significant effect of acetate on the oxidation of the labelled substrate to $^{14}CO_2$. This was probably due to a tendency towards a slightly decreased uptake of β-hydroxybutyrate in the presence of acetate in these experiments. The incorporation of ¹⁴C-label into lipids was not changed by acetate. Neither was there any change in radioactivity in the PCA-extract of the muscle tissue.

The complete oxidation of 1 mol of β-hydroxybutyrate requires 4.5 mol of O_2. Therefore, with the known rates of oxidation of β-hydroxybutyrate (Table II) and the total oxygen consumption (Table I) it can be calculated that the combustion of ¹⁴C β-hydroxybutyrate to $^{14}CO_2$ accounted for about 8% of the total oxygen consumption by the muscle tissue (Table II). In the presence of acetate this figure was decreased to 4%.

. The effect of β-hydroxybutyrate on the metabolic fate of U-^{14}C acetate in the perfused hind-quarter of the rat.

s to medium	Acetate uptake (μmol/h g et weight)	Incorporation of label into		Oxygen consumption due to oxidation of ^{14}C-acetate to $^{14}CO_2$ (%)[d]	Recovery of added label ( )
		$^{14}CO_2$	Lipids		
		(μmol of acetate/h g wet weight)			
ate, 2 mM[b] ion	8.3 ± 0.4 (6)	3.17 ± 0.22 (6)	0.12 ± 0.04 (6)	20.6 ± 1.7 (5)	91 ± 6
ate, 2 mM ion xybutyrate M	7.3* ± 0.2 (6)	2.75 ± 0.11 (6)	0.04* ± 0.01 (6)	18.6 ± 0.6 (6)	98 ± 3

are expressed as mean ± S.E. ith the number of observations in parentheses.
ental conditions see Table I and Methods.
ection has not been made for re-oxidation of NADH by lactate dehydrogenase.
taken from Karlsson et al. (1976).
ficantly different from perfusion without β-hydroxybutyrate, p 0.05

nce of β-h droxybutyrate on ^{14}C-acetate metabolism

as found previously (Karlsson et al 1975), about 40 % of the total uptake of ^{14}C te was oxidized to $^{14}CO_2$ and H_2O (Table III). A minor portion of ^{14}C-label was found ids, hile bout 45 of the label taken up was found in the PCA-extract of the freeze red muscle. The addition of β-hydroxybutyrate caused a slight but not statistically ficant decrease in ^{14}C-acetate oxidation (Table III). This was due to the decrease in the uptake of acetate in the presence of β-hydroxybutyrate in these experiments. Thus, lation to the total acetate uptake the portion of acetate that was oxidized was unaltered n β-hydroxybutyrate was added (Table III). In the presence of β-hydroxybutyrate the rporation of label from ^{14}C-acetate into muscle lipids was reduced to about one third he control alue (Table III).

he oxidation of ^{14}C-acetate to CO_2 accounted for about 20 % of the total oxygen consumpt and this figure was not altered by the addition of β-hydroxybutyrate (Table III).

ctate and pyruvate concentrations and L/P atios in the muscle tissue and the medium

the presence of acetate and β-hydroxybutyrate or both the [lactate]/[pyruvate] (L/P) io was increased in the muscle tissue, as compared to the control value (Table IV). This ect was caused by significantly increased lactate and decreased pyruvate contents when bstrates were added to the standard medium (T ble IV). N similar changes occurred the medium.

The initial concentration ratio between β-hydroxybutyrate and acetoacetate in the medium as about 100. This ratio was significantly lower at the end of the perfusion, mainly due to e uptake of β-hydroxybutyrate from the medium (Table IV).

TABLE II. The effect of acetate on the metabolic fate of D L 3-hydroxy(3- ¹C)butyrate in the perfused hind-quarter of the rat.

Additions to standard medium	β hydro y-butyrate uptake (μmol/h g wet weight)	Incorporation of label into		Oxygen consumption due to oxidation of ^{14}C β-hydroxybutyrate to $^{4}CO_2$ (%)[I]	Recovery of added label ( )
		$^{14}CO_2$ (μmol of β-hydroxybutyrate/h g wet weight)	Lipids		
^{14}C β hydroxybutyrate 0.75 mM	0 97±0.16 (6)	0.45 ±0.03 (6)	0.033±0.004 (6)	8.1 ±0 4 (6)	98_2
^{14}C β-hydroxybutyrate 0.75 mM Acetate mM + infusion	0.80±0.13 (6)	0.27* ±0 02 (6)	0.029±0.008 (6)	4 1* ±0.4 (6)	94±1

Results are expressed as mea ± S.E. with the number of observations in parentheses.
Experimental conditions, see Table I and Methods.
[I] Correction has not been made for re-o idation of NADH by lactate dehydrogenase.
Significantly different from perfusion without acetate, $p < 0.01$

Acetate alone did not significantly affect the oxygen consumption by the perfused tissue (Table I) β-hydroxybutyrate lowered the oxygen consumption as compared to the situation with acetate and this lowering was restored to the control value by the addition of acetate.

In the control experiments, when no substrate was added to the standard medium, the lactate concentration showed only small changes during the perfusion When acetate or β-hydroxybutyrate or both were added to the medium there was a net production of lactate by the perfused tissue (Table I)

Influence of acetate on C β-hydroxybutyrate metabolism

The β-hydroxybutyrate taken up from the medium was readily oxidized in the perfused muscle. Thus, about 50% of the C-label taken up was found in $^{4}CO_2$, while only small amounts were found in lipids in the chloroform extract of the muscle tissue (Table II) About 40% of the total uptake of β-hydroxybutyrate was found as water-soluble metabolites in the PCA-extract of the muscle tissue. When acetate was added the oxidation of ^{14}C-β-hydroxybutyrate was significantly decreased (Table II) However in relation to the total uptake of β-hydroxybutyrate from the medium there was no statistically significant effect of acetate on the oxidation of the labelled substrate to ^{4}CO This was probably due to a tendency towards a slightly decreased uptake of β-hydroxybutyrate in the presence of acetate in these experiments. The incorporation of ^{4}C label into lipids was not changed by acetate. Neither was there any change in radioactivity in the PCA-extract of the muscle tissue

The complete oxidation of 1 mol of β-hydroxybutyrate requires 4 5 mol of O_2. Therefore, with the known rates of oxidation of β-hydroxybutyrate (Table II) and the total oxygen consumption (Table I) it can be calculated that the combustion of ^{4}C β-hydroxybutyrate ^{14}CO accounted for about 8 of the total oxygen consumption by the muscle tissue (Table II). In the presence of acetate this figure was decreased to 4 .

[illegible]	Pyruvate	L/P	β-OH-but/AcAc
0.14 (5)	0.11 ± 0.02 (5)	20.7 ± 4.4 (5)	—
0.13 (6)	0.13 ± 0.01 (6)	18.2 ± 1.4 (6)	—
0.23 (8)	0.14 ± 0.01 (8)	16.1 ± 2.1 (8)	24.8 ± 6.9 (6)
0.13 (9)	0.12 ± 0.01 (9)	19.2 ± 1.7 (9)	19.5 ± 3.5 (10)

[illegible]	P	ATP / ADP P	ADP² / ATP AMP
1.5 (6)	16.0 ± 1.2 (6)	0.46 ± 0.03 (6)	3.5 ± 2.5 (6)
1.6 (5)	15.1 ± 1.6 (6)	0.51 ± 0.07 (6)	5.3 ± 1.8 (5)
± 1.2 (7)	12.8[a] ± 0.3 (7)	0.60[a] ± 0.02 (7)	2.7 ± 0.2 (7)
4.8 (8)	14.7[b] ± 0.7 (8)	0.54 ± 0.04 (8)	2.8 ± 0.7 (8)

The ratio [ADP]²/[ATP]·[AMP], which represents the mass action ratio of the adenylate kinase reaction, was not significantly altered by the presence of β-hydroxybutyrate or acetate or both (Table V).

Discussion

It was found in the present investigation that the total uptake of β-hydroxybutyrate by the perfused rat hind-quarter (Table I) was slightly higher than that found by Ruderman *et al.* (1971) and Ruderman and Goodman (1973) with the same experimental technique. We found, using radioactively labelled β-hydroxybutyrate, that about 45% of the substrate taken up from the perfusion medium was oxidized to CO_2 and H_2O (Table II). Similar results have been found by Balasse and Havel (1971) for acetoacetate oxidation in peripheral tissues. In addition, Hagenfeldt and Wahren (1968) found that about 25% of β-hydroxybutyrate taken up by the human forearm was oxidized to CO_2. Furthermore, the contribution

TABLE IV The effect of acetate or β-hydroxybutyrate or both on the concentrations of lactate and pyruvate and the [lactate]/[pyruvate] (L/P) ratio in the medi m and the muscle tissue of the perfused rat hind-quarter at the end of the perfusion. The [β-hydroxybutyrate]/[acetoacetate] (β-OH but/AcAc) ratio in the medium at the end of the perfusion is also shown.

Additions to standard medium	Muscle tissue		
	Lactate (μmol/g wet weight)	Pyruvate	L/P
Control[I]	1 15 ± 0.20 (6)	0.14 ± 0.0. (6)	7 6 ± 1 4
Acetate, 2mM[I] + infusion	2.21 ± 0.47 (4)	0.08[a] ± 0.01 (6)	27.4[b] ± 1 4
β-hydro ybutyrate 0.75 mM	1 73[a] ± 0.15 (7)	0.09[a] ± 0.01 (7)	20.3[b] ± 1 1
β-hydroxybutyrate 0 75 mM Acetate 2mM + infusion	.30[a] ± 0.26 (8)	0.09[a] ± 0.01 (8)	30 2[b] ± 3.1

Results are expressed as mean ± S E. with the number of observations in parentheses.
Experimental conditions see Table I and Methods.
[I] Data taken from Karlsson *et al.* (1976 a).
[a] *Significantly different from control, $p < 0.05$*
[b] *As for a, $p < 0.01$*
Significantly different from perfusio with β-hydro ybutyrate without acetate, $p < 0.05$.

TABLE V The effect of acetat or β-hydroxybutyrate or both on the concentrations of adenine-nucleotides, creatine-phosphate and inorganic phosphate and the energy-charge in the muscle tissue of the perfused hind-quarter f the rat at the end of the perfusion.

Additions to standard medium	ATP	ADP	AMP (μmol/g et weight)
Control[I]	5.01 ± 0.31 (6)	0.70 ± 0.05 (6)	0 0.4 ± 0.006 (6)
Acetate, 2mM[I] + infusion	5.33 ± 0 16 (6)	0.77 ± 0 09 (6)	0.026 ± 0 004 (6)
β-hydroxybutyrate 0.75 mM	4.87 ± 0.21 (7)	0 64 ± 0.01 (7)	0.034 ± 0 003 (7)
β-hydroxybutyrate 0 75 mM Acetate, 2mM + infusion	5 06 ± 0 19 (8)	0.65 ± 0.01 (8)	0 037[a] ± 0 004 (8)

Results are expressed as mean ± S.E. with the number of observations in parentheses.
Experimental conditions see T ble I and Methods.
[I] Data taken from Karlsson *et al* (1976).
Significa tly different from control, $p < 0.05$
[b] Significantly different from perfusion with β-hydroxybutyrate without acetate $p < 0.05$.
As for a, $p < 0.01$

Tissue concentrations of adenine-nucleotides creatine-phosphate and inorganic phosphate and the "energy-charge" in the muscle tissue

The tissue content of high-energy phosphate compounds (ATP ADP and CP) was not changed by the addition of acetate or β-hydroxybutyrate or both to the medium, as compared to the control value (Table V). However in the presence of acetate and β-hydroxybutyrate there was a statistically significant increase in AMP as compared to the control value (Table V) In addition, *in the presence of β-hydroxybutyrate the tissue content of P_i was* decreased, as compared to the control value. This effect caused an increase in the "phosphate potential" ([ATP]/[ADP]. [P_i]) (Atkinson 1969) The increase in the "phosphate potential" caused by β-hydroxybutyrate, *was restored to the control value by the addition of acetate*

and inorganic phosphate do not give any clear-cut answer how these compounds are involved in the metabolic interaction between β-hydroxybutyrate and acetate (Table V).

During ethanol oxidation in intact animals the redox-state, measured as the concentration ratio between lactate and pyruvate or between β-hydroxybutyrate and acetoacetate, is increased in the liver tissue (Forsander 1970). This change is followed by alterations in several metabolic steps in the liver. The changes in the hepatic redox-state are generally reflected by similar changes in the hepatic venous blood and the increased redox-level is "exported" to extrahepatic tissues, carried in the blood stream (Forsander 1970, Fellenius et al. 1973 b). The present results demonstrate that, in spite of an inhibited oxidation of β-hydroxybutyrate, acetate did not impair the readjustment of the ratio [β-hydroxybutyrate]/[acetoacetate].

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References

ADAM, H. Adenosin-5-diphosphat und Adenosin-5-monophosphat. In: *Methoden der Enzymatischen Analyse*. Ed. by H.-U. Bergmeyer. Verlag Chemie, Weinheim/Bergstr. 196..

ATKINSON, D. E. In: *Metabolic roles of citrate*. Ed. by T. W. Goodwin, Academic Press, London, 1969.

BAGINSKI, E. S., P. P. FOA and B. ZAK, Glucose-6-phosphatase. In: *Methoden der Enzymatischen Analyse*. Ed. by H.-U. Bergmeyer. Verlag Chemie, Weinheim/Bergstr. 1962.

BALASSE, E. O. and R. J. HAVEL, Evidence for an effect of insulin on the peripheral utilization of ketone bodies in dogs. *J. clin. Invest.* 1971. 50. 801–813.

BASSENGE, E., V. E. WENDT, P. SCHOLLMEYER, G. BLÜMCHEN, S. GUDBJARNASON and R. J. BING, Effect of ketone bodies on cardiac metabolism. *Amer. J. Physiol.* 1965. 208. 162–168.

BERGMEYER, H.-U. and H. MÖLLERING, Acetat. Bestimmung mit vorgeschalteter Indikatorreaktion. In: *Methoden der Enzymatischen Analyse*. Ed. by H.-U. Bergmeyer. Verlag Chemie, Weinheim/Bergstr. 1970.

BARTON, R. N., The inter-conversion and disposal of ketone bodies in untreated and injured post absorptive rats. *Biochem. J.* 1973. 136. 531–543.

DOMANSKI, A., D. R. LINDSAY and B. P. SETCHELL, Blood flow and substrate uptake and oxidation in the hind limb muscles of sheep. *J. Physiol.* (Lond.) 1974. 242. 28–29 p.

FELLENIUS, E. and K. H. KIESSLING, Effect of ethanol on fatty acid oxidation in the perfused livers of starved, fed and fat-fed rats. *Acta chem. scand.* 1973. 27. 2781–2790.

FELLENIUS, E., G. BENGTSSON and K. H. KIESSLING, The influence of ethanol-induced changes of the glycerophosphate level on hepatic triglyceride synthesis. *Acta chem. scand.* 1973. 27. 2893–2901.

FELLENIUS, E., U. BOČARKOVA and K. H. KIESSLING, Metabolic changes induced by ethanol in muscle and liver tissue of the rat in vivo. *Acta chem. scand.* 1973. 27. 2361–2366.

FORSANDER, O. A. Influence of ethanol on the redox state of the liver. *Quart. J. Stud. Alcohol* 1970. 31. 550–570.

GOODMAN, M. N. and N. B. RUDERMAN, Ketone-body metabolism in perfused skeletal muscle. *Diabetes* 1972. 21. 343.

HAGENFELDT, L. and J. WAHREN, Human forearm metabolism during exercise. III. Uptake, release and oxidation of β-hydroxybutyrate and observations of β-hydroxybutyrate/acetoacetate ratio. *Scand. J. clin. Lab. Invest.* 1968. 21. 314–320.

HOHORST, H. J., F. H. KREUTZ and T. BÜCHER, Über Metabolitgehalte und Metabolit-Konzentrationen in der Leber der Ratte. *Biochem. Z.* 1959. 332. 18–46.

KARLSSON, N., E. FELLENIUS and K. H. KIESSLING, The metabolism of acetate in the perfused hind-quarter of the rat. *Acta physiol. scand.* 1975. 93. 391–400.

KARLSSON, N., E. FELLENIUS and K. H. KIESSLING, Influence of acetate on glucose metabolism in the perfused hind-quarter of the rat. *Acta physiol. scand.* 1976 a. 98. 347–355.

of β-hydroxybutyrate oxidation to the oxidative metabolism in the perfused muscle w only about 8% (Table II) The low utilization of this substrate was probably due to the low activity of β-hydroxybutyrate dehydrogenase in skeletal muscle (Williamson and He 1970) The low activity of this enzyme is demonstrated by our finding of a slow establi ment of an equilibration in the medium of the concentration ratio between β-hydroxybu rate and acetoacetate. The ratio of 20-25 found at the end of the perfusion (Table I) should be compared with a ratio of 1-2 in rat blood at equilibration (Williamson and He 1970) Our finding of a relatively low contribution of β-hydroxybutyrate oxidation to th total oxidative metabolism in the perfused rat hindquarter at rest (Table II) is suppor by those of Ruderman *et al* (1971) with the same experimental technique of Domand *et al* (1974) in sheep hindlimb and of Barton (1973) in the intact rat *in vivo*

The addition of acetate to the perfusion medium caused a significant decrease in ^{14}C β-hydroxybutyrate oxidation (Table II) Since there was a tendency towards a decreased to uptake of β-hydroxybutyrate by acetate there was no statistically significant effect of aceta if the oxidation of β-hydroxybutyrate is related to the total uptake of β-hydroxybutyra from the medium (Table II) However the oxygen consumption due to oxidation of β hydroxybutyrate, was significantly decreased by acetate. Thus, as may be seen from Table II acetate decreased the contribution of β-hydroxybutyrate oxidation to the energy metabolis of the muscle tissue. This suggests that in our experiments β-hydroxybutyrate oxidation w impaired by the addition of acetate. Ruderman *et al* (1971), Goodman and Ruderman (1972) and Ruderman and Goodman (1973) found that the addition of octanoate or oleat to the perfusion medium did not affect the uptake of acetoacetate in the perfused rat hind quarter However it is not clear from these reports whether there were any effects of th added fatty acids on the oxidation of acetoacetate. It should be pointed out that at com parable concentrations acetoacetate is utilized more rapidly than β-hydroxybutyrate (Ruder man *et al* 1971 Ruderman and Goodman 1973) The pattern of interaction between aceto acetate and acetate might therefore, be different due to the high rate of utilization of aceto acetate, as compared to β-hydroxybutyrate. In accordance with our findings with β hydroxybutyrate in rat skeletal muscle Lefèvre *et al* (1970) found no effect of acetate on the uptake of acetoacetate in the rat diaphragm.

Although there was a slight decrease in acetate uptake by β-hydroxybutyrate we did no find any effect of β-hydroxybutyrate on the oxidation of acetate or the oxygen consumptio due to oxidation of acetate to CO_2 (Table III) This suggests that acetate is preferred a oxidative substrate to β-hydroxybutyrate at the substrate concentrations used in the pre sent study Little *et al* (1970) found that infusion of β-hydroxybutyrate in dogs *in viv* caused a decrease in the uptake of free fatty acids in the resting skeletal muscle and th myocardium Olson (1962) and Bassenge *et al* (1965) found that acetoacetate had a simila effect in the perfused rat heart The differences between the results obtained in this stud and those cited above, concerning the interaction between ketone bodies and free fatty acids, might well be explained by differences in the metabolism of the tissues studied, the ketone body used (β-hydroxybutyrate or acetoacetate) and the concentration of the keton bodies and the short or long-chain free fatty acid

The results of analysis of the redox substrates, adenine-nucleotides, creatine phosphat

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Training Induced Changes in the Subgroups of Human Type II Skeletal Muscle Fibres

By

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Intense endurance training causes no change in muscle fibre type distribution in neither animals nor humans, when fibres are classified into 2 main groups according to contractile properties (Barnard *et al.* 1970, Gollnick *et al.* 1973). On the other hand, an increase in the percentage of the high oxidative at the expense of the low oxidative fast twitch fibres has been reported to occur in animals (Barnard *et al.* 1970). In endurance trained humans, the fast twitch (type II) muscle fibre population has been found almost exclusively to consist of high oxidative (type IIA) fibres (Jansson 1975, Nygaard Jensen 1976). Whether this predominance of type IIA fibres is hereditary or induced by training is not known.

The aim of the present study was to analyze muscle fibre type distribution, with special reference to the subgroups of type II fibres, before and during a period of intense endurance training.

Material and Methods

12 healthy volunteers (age 20–23 years, weight 57–110 kg and height 1.60–1.90 m) participated in the study. They were all fully informed of the risks and discomfort associated with the expts. before they volunteered to participate. All had sedentary occupations and none had been engaged in any regular physical training during the preceding year.

The subjects trained for 8 weeks by pedalling a bicycle ergometer on average 30 min a day, 4 times a week. The average work intensity corresponded to 81 % $\dot{V}O_2$ max (± S.E. 1.1; range 71–88 %). Before and after the training period maximal oxygen uptake ($\dot{V}O_2$ max) was determined, with the subjects pedalling a bicycle ergometer, and muscle biopsies (20–40 mg each) were taken from the quadriceps femoris muscle (vastus lateralis), using the needle biopsy technique (Bergström 1962). On 5 of the subjects this procedure was also followed after 1, 2, 3, and 5 weeks of training.

On testing days the subjects always came to the laboratory in the morning, after a light breakfast. All tests and sampling were performed about 72 h after the last training session.

A detailed description of the maximal oxygen uptake determinations is given elsewhere (Henriksson and Reitman 1976). The muscle samples were mounted in embedding medium (Ames OCT), frozen in isopentane cooled with liquid nitrogen, and stored at −80°C before analysis. Serial transverse sections (10 µm) were cut with a microtome at −20°C, and stained histochemically for myofibrillar adenosine triphosphatase (ATPase) by incubation in a glycine buffer at pH 9.4, after acid or alkaline preincubation in either acetate or glycine buffer. To classify fibres into two main groups, type I and type II, and to identify type IIC fibres (Dubowitz and Brooke 1973), preincubation at pH 4.4 (5 min, 21°C) and at pH 10.3 (8 min, 37°C) was used (Brooke and Kaiser 1969). For classification into subgroups IIA and IIB (Brooke and Kaiser 1970) preincubations at both pH 4.6 (30 s, 21°C) and pH 4.8 (5 min, 21°C) were used.

In most sections, a small number of type II fibres was stained in an intermediate fashion, and could not with certainty be categorized as either type IIA or IIB. On the average these fibres made up 7 % (range 0–21) of the total number of type II fibres, with no systematic variation during the course of the training period. For further treatment of the data half of these fibres in each section were considered as type IIA and half as type IIB.

The significance of intraindividual differences was tested using the paired Student's *t*-test.

KARLSSON, N E. FELLENIUS and K H KIESSLING, Influence of acetate on the metabolism of palmi in the perfused hind-quarter of the rat. *Acta physiol scand.* 1977 In press.

KREBS, H A. und K. HENSELEIT Untersuchungen über die Harnstoffbild ng im Tierkörper *Hoppe-Seyle Z. physiol Chem* 193.. *210* 33–66.

LAMPRECHT W und P STEIN, Creatin phosphat. In *Methode der Enzymatischen Analyse* Ed. by H-U Bergmeyer Verlag Chemie, Weinheim/Bergstr., 1962.

LAMPRECHT W und J TRAUTSCHOLD, Adenosine-5-triphosphate. Bestimmung mit Hexokinase u Zwischenferment. In *Methoden der Enzymatische Analyse* Ed by H U Bergmeyer Verlag Chem Weinheim/Bergstr., 1962.

LEFÈVRE, A., H ADLER and C. S LIEBER, Effect of ethanol on ketone metabolism. *J clin. Invest.* 197 *49* 1775–178..

LITTLE, J R., M GOTO and J J SPITZER, Effect of ketones o metabolism of FFA by dog myocardium and skeletal muscle *in vivo Amer J Physiol.* 1970. *219* 1458–1463

LUNDQUIST F Production and utilization of free acetate in man. *Nature* (Lond.) 1962. *193.* 579–580

LUNDQUIST F., L. SESTOFT S. E. DAMGAARD, J P CLAUSEN and J TRAP-JENSEN, Utilization of aceta in the human forearm during exercise after ethanol ingestion. *J clin. Invest* 1973. *52* 3231 3235.

MAJCHROWICZ, E., Metabolic correlates of ethanol acetaldehyde, acetate and methanol in humans and animals. I *Biochemical pharmacology of ethanol* Ed by E. Majchrowicz. Adv Exp. Med. Biol vol 56. Plenum Press, New York and London, 1975

MELLANBY J und D H WILLIAMSON, Acetacetat. I *M thoden der Enzymatischen Analyse* Ed. by H-U Bergmeyer Verlag Chemie, Weinheim/Bergstr 1970

OLSON R. E., Effect of pyruvate and acetoacetate on the metabolism of free fatty acids by the perfused rat heart. *Nature* (Lond) 1962. *195* 597 598.

RUDERMAN, N B., C. R S. HOUGHTON and R. HEMS, Evaluation of the isolated perfused rat hind-quarter fo the study of muscle metabolism. *Biochem. J* 1971 *124* 639–651

RUDERMAN, N B. and M N GOODMAN, Regulation of ketone body metabolism in skeletal muscle. *Amer J Physiol.* 1973 *224* 1391 1397

WILLIAMSON J R., R SCHOLZ, E. T BROWNING, R. G THURMAN and M H FUKAMI, Metabolic effects of ethanol in perfused rat liver *J biol Chem* 1969 *244* 5044–5054

WILLIAMSON, D H. nd R. HEMS, Metabolism and function of ketone bodies. I *Essays in cell metabolism* Ed by W Bartley H L. Kornberg and J R Quayle. Wiley Interscince, London. New York, Sydney Toronto 1970.

WILLIAMSON D H and J MELLANBY D-(−)-3-Hydroxybutyrat. In *Methoden der Enzymatischen Analyse* Ed by H U Bergmeyer Verlag Chemie Weinheim/Bergstr 1970.

WOLLENBERGER, A., O RISTAU und G SCHOFFA, Eine einf che Technik der extrem schnellen Abkühlung grösserer Gewebestucke. *Pflüg Arch ges Physiol* 1960. *270* 399–41..

rentage distribution of type IIA and IIB fibres was in the same range as that reported by edberg and Jansson (1976) for 68 sixteen year-old boys (32% IIA and 13% IIB fibres).

It is worth emphasizing that the classification of the type II fibres into the subgroups IIA nd IIB in this study is based on differences in the pH sensitivity of the myosin ATPase. ccording to Brooke and Kaiser (1970) this difference is related to the reactivity of sulf ydryl groups of the myosin molecule. Thus it is conceivable that the observed changes ndeed indicate a change in the structure of the myosin molecule.

T give rise to conversion of type IIB to IIA fibres it is likely that, during the training e type IIB fibres are activated much more frequently than in the pre-training situation. ctivation of type IIB fibres at exercise intensities comparable to those used in our training gime are in agreement with the results of Andersen and Sjøgaard (1976).

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References

NDERSEN, P. and G. SJØGAARD, Selective glycogen depletion in the subgroups of type II muscle fibres during intense submaximal exercise in man. *Acta physiol. scand.* 1975. 96. 26A.

ARNARD, R. J. V. R. EDGERTON and J. B. PETER, Effect of exercise on skeletal muscle. I. Biochemical and histochemical properties. *J. appl. Physiol.* 1970. 28. 762–766.

BERGSTRÖM, J. Muscle electrolytes in man. *Scand. J. clin. Lab. Invest.* Suppl. 68. 1962.

BROOKE, M. H. and K. K. KAISER, Some comments on the histochemical characterization of muscle adenosine triphosphatase. *J. Histochem. Cytochem.* 1969. 17. 431–432.

BROOKE, M. H. and K. K. KAISER, Three "myosin ATPase" systems. The nature of their pH lability and sulfhydryl dependence. *J. Histochem. Cytochem.* 1970. 18. 670–672.

DUBOWITZ, V. and M. H. BROOKE, *Muscle biopsy. A modern approach.* Saunders. London. 1973.

GOLLNICK, P. D. R. B. ARMSTRONG, B. SALTIN, C. W. SAUBERT IV, W. L. SEMBROWICH and R. E. SHEPHERD, Effect of training on enzyme activity and fiber composition of human skeletal muscle. *J. appl. Physiol.* 1973. 34. 107–111.

HEDBERG, G. and E. JANSSON, Skelettmuskelfiberkomposition, kapacitet och intresse för olika fysiska aktiviteter bland elever i gymnasieskolan. *Pedagogiska Rapporter.* Umeå, nr. 54, 1976.

HENRIKSSON, J. and J. S. REITMAN, Time course of changes in human skeletal muscle succinate dehydrogenase and cytochrome oxidase activities and maximal oxygen uptake with physical activity and inactivity. *Acta physiol. scand.* 1977. 99. 91–97.

JANSSON, E. Type II fibres in human skeletal muscle. Biochemical characteristics and distribution. *Acta physiol. scand.* 1975. 95. 47A.

NYGAARD JENSEN, E. Adaptational changes in human skeletal muscle with different levels of physical activity. *Acta physiol. scand.* 1976. Suppl. 440. 291.

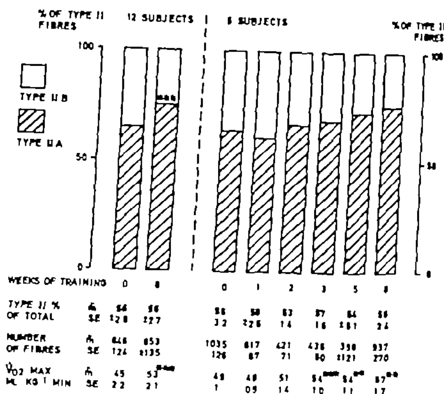


Fig. 1 Change in type II subgroups as percentage of the total number of type II fibres (type IIC fibres not included). In the lower part of the figure, per cent distribution of type II fibres, number of fibres counted, as well as maximal oxygen uptake relative to body weight is given. Significant differences from pre-training values are indicated ($p<0.05$ $p<0.01$ $p<0.001$).

Results

Maximal oxygen uptake ($\dot{V}_{O_2}$ max) The initial mean $\dot{V}_{O_2}$ max (12 subjects) was 45 ml $kg^{-1} \times min$ (b wt 74 4 kg) and increased by 18% to an average of 53 ml kg⁻ min (b wt. 74 6 kg) as a result of the training ($p<0.001$). Individual improvement varied from 8 % to 37%. $\dot{V}_{O_2}$ max during the course of the training period (5 subjects) is given in Fig 1. Also for these subjects body weight was unchanged with training (67.3 kg before *vs* 67.9 kg after)

Fibre type distribution For the whole group fibre distribution was 41% type I 37 % type IIA, and 19% type IIB before as compared to 43% type I 42% type IIA and 14% type IIB after the training period. The observed differences in both type IIA and IIB percentages were statistically significant ($p<0.05$). In order to focus on the type II population the number of type IIA fibres was also calculated in per cent of the total number of type II fibres. Expressed in this way the values are 65% before *vs* 75% after training ($p<0.001$) The time course of the changes, for 5 subjects, is given in Fig. 1 The percentage of type IIC fibres did not change significantly with training (2.6% before *vs* 1.2% after training)

Discussion

The present results indicate a gradual conversion of a part of the type IIB fibre population into type IIA fibres in response to an endurance training programme. The pre-training per

ntage distribution of type IIA and IIB fibres was in the same range as that reported by edberg and Jansson (1976) for 68 sixteen year-old boys (32% IIA and 13% IIB fibres).

It is orth emphasizing that the classification of the type II fibres into the subgroups IIA d IIB in this study is based on differences in the pH sensitivity of the myosin ATPase. ccording to Brooke and Kaiser (1970) this difference is related to the reactivity of sulf ydryl groups of the myosin molecule. Thus it is conceivable that the observed changes deed indicate a change in the structure of the myosin molecule.

To give rise to a conversion of type IIB to IIA fibres it is likely that, during the training e type IIB fibres are activated much more frequently than in the pre-training situation. ctivation of type IIB fibres at exercise intensities comparable to those used in our training gime are in agreement with the results of Andersen and Sjøgaard (1976).

his study was supported by the Danish Natural Science Research Council.

References

NDERSEN, P. and G. SJØGAARD, Selective glycogen depletion in the subgroups of type II muscle fibres during intense submaximal exercise in man. *Acta physiol. scand.* 1975. 95. 26A.

ARNARD, R. J., V. R. EDGERTON and J. B. PETER, Effect of exercise on skeletal muscle. I. Biochemical and histochemical properties. *J. appl. Physiol.* 1970. 28. 762–766.

ERGSTRÖM, J. Muscle electrolytes in man. *Scand. J. clin. Lab. Invest.* Suppl. 68. 1962.

ROOKE, M. H. and K. K. KAISER, Some comments on the histochemical characterization of muscle adenosine triphosphatase. *J. Histochem. Cytochem.* 1969. 17. 431–432.

ROOKE, M. H. and K. K. KAISER, Three "myosin ATPase" systems: The nature of their pH lability and sulfhydryl dependence. *J. Histochem. Cytochem.* 1970. 18. 670–672.

DUBOWITZ, V. and M. H. BROOKE, *Muscle biopsy. A modern approach.* Saunders, London, 1973.

GOLLNICK, P. D., R. B. ARMSTRONG, B. SALTIN, C. W. SAUBERT IV, W. L. SEMBROWICH and R. E. SHEPHERD, Effect of training on enzyme activity and fiber composition of human skeletal muscle. *J. appl. Physiol.* 1973. 34. 107–111.

GRIMBY, G. and E. JANSSON, Skelettmuskelfiberkomposition, kapacitet och intresse för olika fysiska aktiviteter bland elever i grundskolan. *Pedagogiska Rapporter* Umeå, nr 54, 1976.

HENRIKSSON, J. and J. S. REITMAN, Time course of changes in human skeletal muscle succinate dehydrogenase and cytochrome oxidase activities and maximal oxygen uptake with physical activity and inactivity. *Acta physiol. scand.* 1977. 99. 91–97.

JANSSON, E. Type II fibres in human skeletal muscle: Biochemical characteristics and distribution. *Acta physiol. scand.* 1975. 95. 47A.

NYGAARD JENSEN, E. Adaptational changes in human skeletal muscle with different levels of physical activity. *Acta physiol. scand.* 1976. Suppl. 440. 291.

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Vagal Release of Somatostatin into the Antral Lumen of Cats

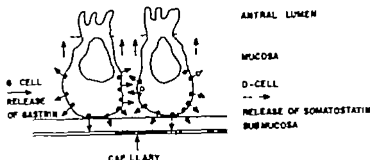
By

KERSTIN UVNÄS-WALLENSTEN SUAD EFENDIC and ROLF LUFT

Large amounts of gastrin 17 have been demonstrated in the gastric juice of cats (Uvnäs-Wallensten 1976, Uvnäs-Wallensten and Rehfeld 1976). Andersson and Nilsson (197 reported that gastrin is present in antral perfusates of dogs. In the cat gastrin is released directly into the antral lumen in parallel with the release of gastrin into the gastric venous outflow Thus electrical vagal stimulation causes a release of gastrin both into the antral lumen and into the gastric venous blood (Uvnäs-Wallensten to be published).

The occurrence of gastrin in the gastric lumen, as well as in the circulation, might be explained as due to gastrin being released diffusely into the interstitial fluid from the G-cells (Fig. 1) Gastrin might then exert local or paracrine effects on neighbouring cells, as well as blood borne remote endocrine effects Somatostatin containing cells are located close to the G-cells in the antral mucosa and both types of cells have similar morphological characteristics. Therefore somatostatin might also be expected to be released into the antral lumen

The only well established mechanism by which the release of gastrin can be inhibited is by low intra-antral pH It has been assumed that the effect of low pH is exerted directly on the G-cells. The presence of somatostatin cells close to the G-cells in the antrum, and its ability to antagonize the release of gastrin (Bloom *et al* 1974 Le Roith *et al* 1975 Rapts *et al* 1975 Uvnäs-Wallensten *et al* in press), suggested to us that the inhibitory effect of low pH might be mediated via a release of somatostatin To test this hypothesis, the antrum of 5 cats was perfused with slightly alkaline or/and acid solutions and vagal stimulations (5 Hz) were performed during the perfusions. Perfusates were assayed for both somatostatin and gastrin immunoreactivity Immediately upon collection, the pH of the samples was adjusted to 7 they were boiled for 15 min and then frozen until the hormone content was determined by radioimmunoassay Since the antrum was perfused at a constant rate (1 ml/min) the output of hormone per minute could be calculated. In all expts. vagal stimulation caused an increased release (100%) of somatostatin into the antral lumen during perfusion with 0 1 M HCl when the output of gastrin was reduced to about 30 %. During perfusion with a slightly alkaline medium, vagal stimulations almost failed to cause a release of somatostatin (7%), but large amounts of gastrin were released instead (100 %) (Table I). A typical experiment is illustrated in Fig. 2. On the average 351 ng of gastrin were released by vagal stimulation during perfusion with phosphate buffer (pH 7 8) while the maximal



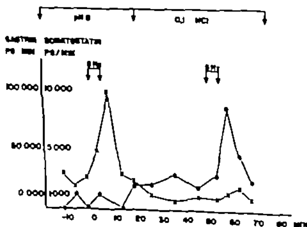
1 Schematic picture of gastrin containing cell (G-cell) and somatostatin containing cell (D-cell). arro illustrate how the tw hormones might be released diffusely from the cells. This model ould ain how the hormones from one typ of hormone producing cells can, at the same time, exert paracrine endocrine actions and also appear in the gastrointestinal lumen

ase of somatostatin, obtained during perfusion with 0 1 M HCl, was only about 7? ng ble I)

Both gastrin and somatostatin can be released by vagal stimulation. The results with a iprocal occurrence of gastrin and somatostatin in the antral lumen suggest that the ibitory effect exerted by HCl on the release of gastrin, might at least partially be due to ocal effect of somatostatin on the G-cells.

The antral gastrin and somatostatin producing cells probably release their hormones into surrounding interstitial tissue, from which the hormones diffuse in all directions. Gastrin s been demonstrated to reach the antral lumen as well as the blood. Until now in re onse to vagal stimulation, somatostatin has only been demonstrated to occur in the antral men. However simultaneous release of the hormone might be expected to occur into the culation

The gastrointestinal mucosa contains a large number of different hormone producing lls. It might therefore be expected, that the hormones produced by these cells also will pass



ig 2 Experiment in which the antrum as perfused with phosphate buffer pH 8 and 0 1 M HCl Vagal stimulation (5 Hz for 5 min) was performed during perfusion with both solutions

T BLE I Release of gastrin (G) and somatostatin (S) i to the antral lumen during perfusion th HCl and/or phosphate buffer pH 7–8. The output of hormone was determined quantitat and expressed in ng. In experiments in which stimulations were performed at both pHs, the output was expressed in of the maximal output (100). Note that the maximal outp gastrin (pH 7–8) was on the a erage 5 times higher than that of somatostat n (pH 1 2)

Exp. no.	pH 1		pH 7–8	
	g	%	ng	
1 G	75	14	525	100
S	46	100	0	0
2 G	118	37	320	100
S	6	100	3	1
3 G	60	38	160	100
S	79	100	3	10
4 G			400	
S			0	
5 G	34			
S	10			
Mean				
G	72	30	351	100
S	18	100		7

into the gastrointestinal lumen. Since many gastrointestinal hormones cannot be meas in the blood, due to methodological difficulties or to too low circulating hormone lev it might even be advantageous to follow the output of these hormones in gastrointest perfusates.

This study was supported by grants from Amundsons fond and Nordisk Insuli fond

References

ANDERSSON S. and G NILSSON Appearance of gastrin in perfusates from the isolated gastric sacs of dogs. *Scand J Gastroent* 1974 9 619–621

BLOOM, S. R., C. H MORTIMER, M O THORNER, G M BESSER, R H LL, A. GOMEZ PAN, U M R R. C. O RUSSEL, D H COY A. J KASTIN and A. V SCHALL Inhibition of gastrin and gastric a secretion by growth hormone release inh biting hormone. *Lancet* 1974 9 1106–1109

LE ROITH D A. J VINIK, S. EPSTEIN P B RON M N O ENITZKY and B L PIMSTONE Somatostatin and serum gastrin in normal subjects and n patients with pernicious anaemia, chronic l er and r disease *S. Af med. J* 1975 49 1601 1604

RAPTIS, S., H C. DOLLINGER, L. VON BERGER W SCHLEGEL, K. E. SCHRÖDER nd E. F PF IFFER. Effe of somatostatin on gastric secretion and gastrin release man. *Digestion* 1975 13 15–26.

UVNÄS WALLENSTEN, K., Gastrin release nd HCl secretion induced by electrical gal stimulation in t cat. *Acta physiol. scand.* 1976 S ppl 438

UVNÄS-WALLENSTEN K., The occurrence of gastri in gastric j ice l tral secretion and l ntral p fusates. S bmitted for p blication in Gastroenterology

UVNÄS-WALLENSTEN, K., S. EFENDIC and R LUFT Inhibition of gally induced ga t release by somat stati in cats. *Hormone and M tabolic R search* 1977 I press.

UVNÄS-WALLENSTEN, K. and J REHFELD, Molecular forms of gastri l tral mucosa, plasma and gas juice duri g gal stimulatio of anaesthetized cats. *Acta ph siol scand.* 1976. 98 17 26.

Acta physiol. scand. 1977. 99. 129–139

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Coacervate-like Membrane Structures and Olfactory Transduction

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Abstract

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Current theories concerning the olfactory transduction process are discussed. A hypothesis is formulated, according to which the olfactory receptor membrane contains regions where it has the structure of a lipid-protein coacervate. Such structures may well occur in living cells. Such a membrane would have the ability to change its permeability in response to odorants and a sensitivity comparable to that of the sense of smell. The model also explains the fact that different receptor cells have different sensitivity patterns towards odorants. The model is consistent with the results of experiments that seek to establish the locus of odorant action.

At present, it is usually accepted that the olfactory receptor cells function essentially as described by Ottoson (1970). According to this view the primary event in smelling is some process whereby odorant molecules, entering the plasma membrane of the cilia or comparable structures of the receptor cell, cause some change in the ionic permeability of this membrane. The nature of this transduction process is unknown. In this paper the model for the transduction process put forward by Bungenberg de Jong and Saubert (1937) is reformulated and reevaluated in the light of new experimental data (Sperber, in press) and present-day knowledge of the physiology of the sense of smell.

The many "theories of smell" do not concern themselves much with how the transduction process actually works. Most theories bypass the question entirely and some make a general assumption (such as "membrane-active substances" or "allosteric proteins") about it, which is not an integral part of the theory. In recent times, only two specific hypotheses about the olfactory transduction mechanism have been developed further than mere suggestions.

Rosenberg *et al.* (1968) suggested that the conductance of carotenoids in the membrane could be changed by the odorant molecules. They showed that the conductance of carotenoid crystals is actually very sensitive to many vapours. Against this attractively clear and

TABLE I Release of gastrin (G) and somatostatin (S) into the a tral lumen during perfusion ith 0.1 HCl and/or phosphate buffer pH 7–8. The output of hormone was determined quantitatively and expressed in ng. In experiments in which stimulations were performed at both pHs, the output was expressed in of the maximal output (100 %). Note that the maximal output gastrin (pH 7–8) was on the a erage 5 times higher than that of somatostatin (pH 1 2).

Exp. no		pH 1 2		pH 7–8	
		ng		ng	
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	S	26	100	3	12
3	G	60	38	160	100
	S	79	100	3	10
4	G			400	
	S			0	
5	G	34			
	S	10			
Mean					
	G	72	30	351	100
	S	28	100	2	7

into the gastrointestinal lumen. Since many gastrointestinal hormones cannot be measured in the blood due to methodological difficulties or to too low circulating hormone levels it might even be advantageous to follow the output of these hormones in gastrointestinal perfusates.

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References

ANDERSSON, S. and G NILSSON, Appearance of gastri in perfusates from the isolated gastric antrum of dogs. *Scand J Gastroent* 1974 9 619–6.1

BLOOM, S. R C. H MORTIMER, M. O THORNER, G M BESSER, R HALL, A GOMEZ PAN, U M R. R. C. G RUSSEL, D. H COY A. J KASTIN and A. V SCHALLY Inhibition of gastrin nd gastric ac secretion by growth hormone release i h biting hormone. *Lancet* 1974 9 1106–1109

LE ROITH, D A. J VINIK S. EPSTEIN, P B son, M N OLKENITZKY nd B L. PIMSTONE, Somatostatin and serum gastrin in normal subject and in patients with pernicious naemia, chronic l er nd ren disease. *S Af med J* 1975 49 1601 1604

RAPTIS, S., H C. DOLLINGER, L. VON BERGER W SCHLEGEL, K E. SCH ÖDER nd E. F PF IFFER, Effect of somatostatin on gastric secretion nd gastrin release in man. *Digestion* 1975 13 15–26.

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UVNÄS-WALLENSTEN, K., The occurrence of ga tri gastric j ice, i tral secretion and in antral perfusates. S bmitted for publication in Gastroenterology

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UVNÄS-WALLENSTEN, K. and J REHFELD, Molecular forms of gastri in tral mucosa, plasma and gastric juice during agal stimulation of anaesthetized cats. *Acta ph iol. scand* 1976. 98 217 226.

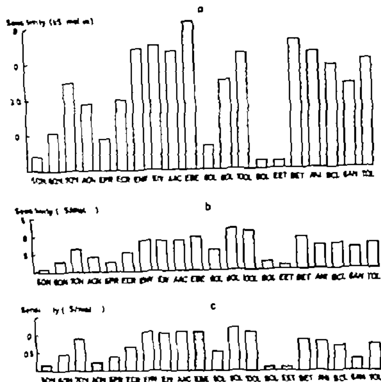


Fig. 1. Sensitivity profiles of three coacervates towards 20 additives. The coacervant was heptanone-2 (a), n-butanol (b) and benzyl alcohol (c). Additives are, from left to right, pentanone, iso-hexanone, heptanone, acetophenone, ethyl propionate, ethyl crotonate, ethyl-n-valerate, ethyl isovalerate, amyl acetate, ethyl benzoate, hexanol, octanol, decanol, benzyl alcohol, ether dibutyl ether, anisole, benzyl chloride, hexane and toluene. Shaded columns correspond to data of dubious value (see (Sparber, in press)).

summary of these investigations will be presented here, and the results discussed from a physiological point of view with regard primarily to the sense of smell in vertebrates.

Methods

For complete description, see Sparber (in press).

Coacervates were prepared from water, NaCl, egg lecithin, surfactant (dodecyl benzene sulphonate) and "coacervant", a moderately polar substance, which could be varied (9 different coacervants were used). These constituents were mixed stepwise in test tubes, and the coacervate appeared as a layer of liquid on top of a larger volume of water.

Also, 20 different organic compounds were used as "additives", analogous to odorants, which could be included in the coacervate in standardized amounts. The electrical conductivity of the resulting coacervate was measured with standard methods and compared with the conductivity of a similar coacervate with no additive included.

The concentration of Na, K, Ca, Mg, S, P and Cl⁻ in the coacervates were measured with standard methods. The concentration of water was determined with tracer. In some experiments the diffusion in coacervate of two dyes, one hydrophilic and one lipophilic, was followed optically.

simple theory. It may be objected that there is some doubt whether any carotenoids are present in the receptor cells (Takagi and Yajima 1965), and that the conductance of the carotenoids so far investigated is too low.

The hypothesis of Davies and Taylor (1957), the "penetration and puncturing theory", assumes that when odorant molecules adsorbed into the lipid membrane desorb again, they leave a hole in the membrane for some time, through which ions may pass. This theory can predict odour quality to some extent (Davies 1970), but no model membrane has been found which actually shows such properties. Experiments on artificial bilayers have been somewhat disappointing (Cherry *et al.* 1970).

The contribution of colloid chemists Bungenberg de Jong and Saubert to the physiology of smell sprang from their work on 'coacervates'. Coacervates are colloid solutions, whose solvent is generally a solution of salt in water, which have an internal coherence, so that they cannot be diluted, but exist in equilibrium with their solvent without mixing freely with it.

Coacervates of lipids have the property that when foreign substances, especially organic ones, are added, the concentration of the coacervate changes. This may occur with quite small amounts of the added substance. Bungenberg de Jong and Saubert (1937) proposed in general terms that this phenomenon could have some connection with the sense of smell, but they had to be vague, in view of the sparse physiological data available at the time.

In the context of the above picture of olfactory physiology, a "revised coacervate hypothesis" can be formulated.

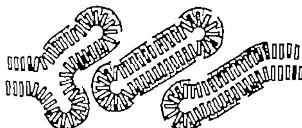
The sensitive membrane of the olfactory receptor cells is assumed to have regions where the membrane lipids take on a coacervate-like structure. When an odorant arrives at the membrane, it enters these regions and there causes the ratio between water, which is electrically conducting, and lipid, which is not, to change so that the ionic permeability changes.

The last step of this reasoning has been supported experimentally: if the concentration of a lipid coacervate is raised through the addition of an organic substance, its electrical conductivity falls (Sperber, in press). The conductivity is, of course, a measure of the permeability of the coacervate to the ions present, in this case mainly sodium and chloride ions.

If the lipid coacervates are to serve as a membrane model, their structure is naturally of major interest. Little is known with certainty about this, but a good working hypothesis has been presented by Booij (1949), according to which the lipid is distributed in the form of flat "sandwich" micelles, essentially pieces of lipid bilayer. The negative surface charge of the micelles is assumed to be nearly neutralized by positive counterions which are unevenly distributed, so that the micelles can come into loose contact with each other at points where their charge is completely neutralized by counterions, but are still kept apart by the electrostatic repulsion between regions where the charge is not completely neutralized. As occlusion water will then be locked between the micelles, this picture explains how a considerable but limited amount of water can be kept in the coacervate.

Even less is known about the mechanism by which foreign molecules influence the conductivity of the coacervate, and available hypotheses are unconvincing. See (Sperber, in press) for a discussion of this problem.

In order to gain more insight into the possibilities of the model, a technically suitable lipid coacervate has been developed and its properties investigated (Sperber, in press). A



g. 2. This figure is intended to give an idea of how small bulge on the membrane, about 20 nm thick, ald contain relatively complex, coacervate-like structure. I is not to be interpreted as showing actual tails. The rectangles denote lecithin molecules (about 2.5 nm in length). The lines denote protein.

The coacervants were uncharged organic compounds with a polar group (alcohol, ketone, ther or ester) and a hydrocarbon chain with 4–8 carbon atoms. There are many biological ompounds of this type, but they are volatile. It is possible that the receptor cells could ynthesize such compounds rapidly enough to keep the receptor membrane in a steady state, out in cells that are specialized for close diffusional contact with the outside air it is not a empting concept. A more attractive thought is that the role of the coacervant is taken over by amino acid residues of the membrane proteins. An amino acid residue with its peptide group is in fact quite similar to the coacervants, except that its mobility is reduced by its being a member of polypeptide chain. This difference may not be important. Double coacervates containing both proteins and lipids exist, although mainly with one-chain lipids rather than lecithin. According to Pankhurst (1949), one kind of double coacervate with gelatin and lauryl sulphate probably has one lauryl sulphate molecule bound to each peptide link in the gelatin.

Therefore, as an extension of the model, the coacervate-like structure in the receptor membrane is assumed to be *complex between lipid and protein*, but retaining the essential characteristics of the protein-free coacervates used in the experiments. This assumption will be used in the following discussion.

Another question is whether there will be space enough in the membrane for a coacervate-like structure. At present, it cannot be determined how small a volume of coacervate will retain the macroscopic properties. It is conceivable that the forces determining changes in coacervates may operate on a mere pore or rift in the membrane. Even a more complex structure, such as sketched in Fig. 2 would only constitute moderate bulge on the membrane. In principle, micellar structures may well occur in membranes this has been discussed by Lucy (1970).

The membrane potential change

Getchell *et al.* (1963) found that the response of a receptor cell to an odour was in most cases an excitation, but inhibition was not uncommon. In general, this has been confirmed by later investigators (for references, see Sensitivity patterns). There is, however some doubt about the physiological relevance of inhibition (Duchamp *et al.* 1974). Also in many cases, inhibition may have been an artifact caused by withdrawal of background odour

TABLE I Traits in the dependence of coacervate sensitivity patterns on the coacervant.

Trait No	Coacervant	Coacervate particularly sensitive to	Coacervate particularly insensitive to	Comments
I	Any alcohol	Alcohols		
II	Benzyl alcohol		Hexane	
III	Heptanone		Ether	
IV	Heptanone		Ethyl propionate	Compared to [illegible] coacervant [illegible] as ethyl propionate or ether
V	Benzyl alcohol	Hexanone		
VI	Heptanone or octanol or hexenol	Ethyl benzoate and anisole and toluene		

Data

For a full presentation see Sperber (in press)

It was found that when an additive is included in a coacervate, the conductivity of the latter falls roughly in proportion to the amount of additive. The proportionality factor the "sensitivity" depends on both additive and coacervant

This dependence was investigated for 9 coacervants and 20 additives. Some of the results are shown in Fig. 1 All the coacervates tended to be more sensitive to additives with long hydrocarbon chains. Beyond this simple rule, the dependence was very complicated. S relatively simple and distinctive traits could be discerned in the material. These are presented in Table I

The optical properties and chemical composition of the coacervates were consistent with the structure pictured by Booij The dye diffusion experiments, however suggested that the lipids may form a continuous, though not static, network rather than micelles.

Comparison between the coacervate model and biological data

In this section, the biological relevance of the revised coacervate model for olfactory transduction will be discussed although sufficient information is not yet available to make a complete evaluation of the model

Conditions for the existence of coacervates in cells

At normal temperatures, the coacervates form spontaneously from their constituents. Of the constituents, water and salt are present in living cells, and lecithin is abundant in the olfactory epithelium (Koyama *et al* 1971), though not exactly identical with egg lecithin.

Dodecyl benzene sulphonate is a nonbiological compound, but it was chosen for technical reasons, and a coacervate will still form if it is exchanged for another anionic surfactant, such as palmitate or lauryl sulphate or if it is excluded altogether (Sperber 1973) Negatively charged lipids, such as fatty acids, cephalin or phosphatidic acid should be able to take its place in a cell membrane

voke the presence of two different types of coacervate in the same membrane, with different on selectivity and different sensitivity patterns to odorants. On the other hand, it is easy show that e.g. ethanol causes an increase in the conductivity of the coacervate. Also, other pid coacervates, e.g. oleate coacervates, often react to additives with a decrease in concentration (Bungenberg de Jong et al. 1938), which ought to mean an increase in conductivity. This is especially the case if the additive is of low molecular weight or is used in comparatively large amounts (most single-cell recordings have been obtained using rather powerful stimuli).

Sensitivity patterns

There are several investigations published where recordings have been made from a number of single receptor cells, while they are stimulated with a number of different odorants. Except for insect "specialist" receptors, such experiments yield bewildering results (Boeckh et al. 1965, Kay 1971, Sass 1972, Holley 1974, Giachetti and MacLeod 1973, Duchamp et al. 1974, Holley et al. 1974, Masson and Friggi 1974, Mustaparta 1975. For further references on vertebrates, see Getchell 1973). The cells have, as a rule, a rather low selectivity and may show similar reactions to odorants that are both chemically and organoleptically dissimilar. Two cells with identical sensitivity patterns are very difficult to find. Of several attempts to find at least rudimentary order in such results, the most convincing is that of Duchamp et al. (1974) on the frog. They found that any single cell tended to give similar responses to all the aromatic compounds among the stimuli.

Some of the observed differences between cells may be caused by factors outside the cells. According to Mozell (1964), a kind of chromatography may occur on the olfactory mucosa, causing different odorants to be concentrated in different parts of the mucosa, so that identical cells in different positions will be expected to show different sensitivity patterns. Also, the position of the cilia in the mucus covering the epithelium should be important. A cell whose cilia lie on the surface of the mucus will be reached by all odorants, while a cell whose cilia lie deep in the mucus, for example a freshly developing cell such as observed by Graziadei and Metcalf (1971), will be reached mainly by odorants with high water solubility.

It is, however, by no means possible to explain all the results in this way, and it is clear that the sensitivity pattern of the receptor membrane itself can vary considerably between cells. This is very likely the basis of the ability to distinguish between smells. It is very important for a hypothesis of receptor cell function to explain the variability of sensitivity patterns, and both theories mentioned earlier make a point of it. Rosenberg et al. (196[illegible]) showed directly that different carotenoids have very different sensitivity patterns. In the "penetration and puncturing theory" there is not such clear evidence, but it is assumed that the sensitivity pattern of a membrane will depend on its viscosity (Davies 1970).

The coacervate hypothesis can also explain the variability of sensitivity patterns, since it has been clearly shown (Sperber, in press) (see also Fig. 1) that *coacervates show different sensitivity patterns*, depending on which coacervant is used. Thus, if the coacervate-like structures in two cells contain different amino acids, the cells will be expected to have different sensitivity patterns. There is no definite limit to the variation possibilities, though it seems that the coacervates so far investigated do not differ as much as receptor cells do.

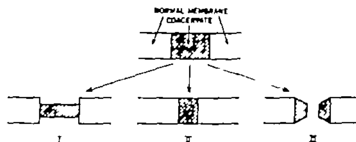


Fig. 3 If coacervate-like structures in a membrane shrink under the influence of odorants, this may contribute, depending on mechanical factors, (I) a permeability increase because of membrane thinning, (II) a permeability decrease, because of channel narrowing, (III) a large permeability increase, because of pore formation.

It is not clear what changes in the permeability of the receptor membrane cause the generator potentials. Attempts have been made to investigate them by ion substitution techniques (Takagi *et al.* 1966, 1968 1969), but the results are doubtful, because the experiments were of such long duration that the thin cilia of the receptor cells may have been considerably damaged by the solutions. Also, single cells were not studied, but the EOG which may contain several components.

Thus, many ion species may be involved in the generator potential To simplify the reasoning, however the following discussion will concern only the roles of sodium and potassium ions.

In other cases, generator potentials are usually caused by an increased permeability to some ion, but not without exception (Tomita 1970) Therefore, the coacervates used in the experiments could be involved in generator potentials in spite of their strong tendency to decreased conductivity in response to additives. Also, they may cause either depolarizing or hyperpolarizing generator potentials, depending on the properties of the normal (non-coacervate) part of the membrane. If the resting potential is assumed to be determined by the relation between sodium and potassium permeabilities, and the coacervate parts are more potassium selective than the normal membrane, an overall permeability decrease in the coacervate parts will result in a decreased potassium selectivity of the whole membrane and a depolarizing generator potential. Conversely if the coacervate parts are less potassium selective than the normal membrane, a hyperpolarizing generator potential will result. Of course, if a selectivity change in the coacervate itself were to accompany the overall permeability decrease, a generator potential would result irrespective of the properties of the normal membrane.

In this connection it should be pointed out that odorants cause not only the conductivity, but also the volume of lipid coacervates to decrease, especially in the more dilute coacervates. If such a volume decrease occurs in coacervate-like structures in a membrane, it may lead to various changes in the permeability depending on mechanical parameters (Fig. 3).

In terms of the coacervate model it is a little more difficult to account for the fact that one cell may show both excitatory and inhibitory responses to different odours unless the inhibitory responses are artifacts or caused by a different mechanism If the coacervate can react to an odorant only with a decrease in permeability (as in (Sper in press)), one must

$$E \quad 61 \log\frac{(25+2)4+(1+2)145}{(25+2)155+(1+2)12} \quad -54.3 \text{ mV}$$

he permeabilities of the coacervate part are decreased by 14% the membrane potential l be

$$E \quad 61 \log\frac{(25+1\ 72)4+(1\quad 1\ 72)145}{(25\quad 1\ 72)155+(1+1\ 72)12} = -56.2 \text{ mV}$$

a change by about 3.5%. It does not seem unreasonable that this could cause an apprecie change in the spike frequency of the cell.

A rough estimate of the amount of odorant required *for the transduction* (the total amount quired will be considerably larger because the normal membrane will also take up odorant) n be calculated as follows:

If the normal membrane has a K conductance of 5 S/m (2 000 Ω cm²), then, under the regoing assumptions, its Na conductance will be 0.2 S/m² and the K and Na conducnce of the coacervate parts will be 0 4 S/m each and the total conductance of the coacere parts 0.8 S per m membrane If the coacervate parts have a conductivity of 1 S/m and re assumed to be 20 10⁻⁹ m thick (*cf* Fig. 2), 1 m of coacervate membrane will have a onductance of

$$\frac{1 \quad 1}{20 \quad 10^{-9}} = 5 \quad 10^7 \text{ S.}$$

As the coacervate parts of 1 m of membrane have a conductance of only 0 8 S, this means that

$$\frac{0\ 8}{5 \quad 10} \quad 1.6 \quad 10^{-8}$$

of the membrane area consists of coacervate. At threshold, only the most superficially situated cilia on the epithelium will contain appreciable amounts of odorant, since their membrane lipids act as a sink for the odorant molecules. Their membranes will have an area comparable to that of the regio olfactoria, *i.e.* of the order of 10⁻⁴ m of which 1 6 10 10 1.6 10⁻ m is coacervate, and contain 1.6 10⁻ 20 10⁻⁹ = 3.2 10⁻ m³ of coacervate As the coacervate contains about 10⁶ lipid, there will be 3.2 10⁻ m³ 3.2 10 cm lipid, weighing about 3.2 10⁻ g, in turn containing 0.06 mmol of odorant per g, the coacervate parts will contain 3.2 10⁻¹⁴ 0.06 1.9 10⁻¹⁵ mmol, *i.e.* of the order of 10⁸ odorant molecules.

The location of the interaction

Some attempts have been made to show that some specific kind of molecules in the membrane act as acceptors for the odorant molecules. Odorants have been shown to interact with various kinds of molecules, but it is still not clear which interactions are essential and which are incidental. There seems, however to be a general feeling that protein molecules are the most likely acceptors.

Spectrometric methods have shown that odorants cause conformation changes in both lipids (Dodd 1971) and proteins (Ash 1969) in the olfactory epithelium. In neither case have the changes been shown to be connected with the transduction process.

In view of the grouping of aromatic odorants found by Duchamp *et al.*, mentioned above it is interesting to note that coacervates where the coacervant has a comparatively long hydrocarbon chain seem to have an increased sensitivity towards aromatic additives (the last in Table 1) At the present stage it is hardly meaningful to try any more detailed comparisons between the sensitivity patterns of receptor cells and coacervates.

Degree of sensitivity

It is very difficult to calculate whether the sensitivity of the coacervates to odorants is high enough to explain the high sensitivity of the sense of smell About such factors as turbulence in the nasal passages ion distribution and permeability of the receptor membrane and the *sensitivity of the action-potential generating mechanism there is at least room for reasonable* guesses. The most troublesome problem is that the receptor cells as a rule are spontaneously active and therefore do not have a definite threshold Instead, the olfactory threshold will ultimately be determined by the ability of the brain to register minute changes in the average firing frequency of the receptors. About this ability we know hardly anything. It may be exceed the corresponding ability of the experimenter studying single cells, since the receptors converge strongly on the higher-order neurons. In spite of these difficulties, the following argument attempts to show that the sensitivity of the coacervates may well suffice.

Choosing decanol as an example, we find the biological threshold for man given as 3 6 *10 molecules per cm of air (Davies and Taylor 1957) According to the same source,* the partition coefficient between the lipid–water interface and air is 10 Thus the threshold concentration in air corresponds to an equilibrium concentration of 3 4 10 molecules per cm in the membrane. As the lipids have about the same density as water this corresponds to about 0 6 mmol of decanol per g lipid It is doubtful whether there will be time under normal conditions for equilibrium to be reached but if we assume that at least 10 of equilibrium is reached in the membrane of the most superficial cilia this means that 0 06 mmoles per g lipid are needed for stimulation

The coacervate where the coacervant was ether showed a sensitivity towards decanol of about 5 kS/mol m.[1] Since the coacervate contained about 0 45 g of lipid (lecithin + surfactant) this means that a concentration of 0 06 mmol per g lipid causes a decrease in conductivity of 0 06 0 45 5 – 0 135 S/m This means that the conductivity of the coacervate decreases by about 14 %, since the coacervate without decanol had a conductivity of nearly 1 S/m.

Assume that the membrane potential of the receptor membrane is determined by its permeability to sodium and potassium ions, which have normal concentrations (Na 145 mM Na_i – 12 mM K – 4 mM K_i – 155 mM). Assume that the normal part of the membrane is 25 times more permeable to K than to Na while the coacervate parts are nonselective Assume that the area and permeability of the coacervate parts are such that they contribute to the total membrane *sodium and potassium permeabilities that are twice the sodium* permeability of the normal part. Then by the Goldman equation the potential of the unstimulated membrane is

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[1] S (siemens) = ohm^{-1}

ves, J T and F H Taylor, Molecular shape, size and adsorption in olfaction. *Proc 2nd. Internat Congr Surf Act* 1957 329–340.
...oo, G. H Studies on olfactory receptor mechanisms. *Biochem J* 1971 *123*. 31P 32P
...xhamp A., M F Revial, A Holley and P MacLeod, Odor discrimination by frog olfactory receptors *Chem Senses and Flavor* 1974 *1* 213–233.
...edman, L and J G Miller, Odor incongruity and chirality *Science* 1971 *172*. 1044–1046.
...theland R C J Y Lettvin, W H. Pitts and A Rojas, Odor specificities of the frog's olfactory receptors In *Olfaction and Taste* Ed Y Zotterman, Pergamon Press 1963. 19–34.
...tchell, T V Analysis of unitary spikes recorded extracellularly from frog olfactory receptor cells and nasal *J Physiol* (Lond) 1973 *234* 533–551
...tchell, M L and R C Gesteland, The chemistry of olfactory reception: stimulus-specific protection from sulfhydryl reagent inhibition. *Proc nat. Acad. Sci.* (Wash) 1972. *69* 1494–1498.
...acchetti, L and P MacLeod Supériorité de pouvoir discriminateur des cellules mitrales comparé celui des récepteurs olfactifs *J Physiol* (Paris) 1973, *66*, 399–407
...oon, D. V and T Wan Ng, Trinitrobenzenesulfonic acid. A possible chemical probe to investigate lipid-protein interactions in biological membranes. *Molec Pharmacol.* 1972, *8* 426–437
...aratadis, P P C and J. F Metcalf Autoradiographic and ultrastructural observations on the frog olfactory mucosa *Z Zellforsch* 1971 *116*, 305–318.
Holley A The discriminating power of the vertebrate olfactory receptors. In *Transduction mechanisms in chemoreceptors*, Ed T M Poynder, Information Retrieval Ltd 1974
Holley A A. Duchamp M. F Revial and A. Juge, Qualitative and quantitative discrimination in the frog olfactory receptors: analysis from electrophysiological data. *Ann. N Y Acad. Sci.* 1974, *237* 102 11
...a R E Olfactory unit potentials and receptor potential responses of Loculus sericata. *Amer J Physiol* 1971 *220* 1481 1487
Koyama, N K Sawada and K Kurihara, Isolation and some properties of plasma membranes from bovine olfactory epithelium. *Biochim biophys. Acta* (Amst) 1971 *241* 42–48.
Luttro, T J P G Guadagni, J Harris, T R. Mon and R Teranishi, Evidence for the difference between the odours of the optical isomers ( )- and ( )-carvone *Nature* (Lond) 1971 *230* 455–4...
Lucy J A The fusion of biological membranes. *Nature* (Lond.) 1970. *227* 815–817
Mason, C and A Fridge, Codage de l'information par les cellules des récepteurs olfactifs de l'antenne de Camponotus vagus *J Insect. Physiol.* 1974 *20* 763–782
Moncrieff, M M., Evidence for sorption as mechanism of the olfactory analysis of vapors. *Nature* (Lond) 1964 *203* 1181–1182
Mustaparta, H., Responses of single olfactory cells in the pine weevil Hylobius abietis L. *J comp Physiol* 1975 *97* 271 290
Ottoson, D Electrical signs of olfactory transducer action. I *Taste and Smell in Vertebrates*, Ed E. Wolstenholme and J Knight, Churchill 1970. 343–356
Pankhurst K G A The influence of size of ions on the binding of amphipathic anions and cations by gelatin *Discuss Faraday Soc* 1949 *6* 52–62
Rosenberg, B T N Misra and R Switzer, Mechanism of olfactory transduction. *Nature* (Lond) 1968 *217* 423–427
Sass, H Das Zusammenspiel mehrerer Rezeptortypen bei der nervösen Codierung von Geruchsqualitäten *Verh dtsch zool Ges* 1972 *66* 198–201
Spitzer, G O Changes in the electrical resistivity of lecithin coacervates as model for olfactory transduction *Acta physiol scand* 1973. *89*, 603–605.
Spitzer, G O Conductivity and structure of group of lipid coacervates. *J Colloid Interface Sci* ...
Takagi, S F and T Yajima, Electrical activity and histological change in the degenerating olfactory epithelium *J gen Physiol* 1965 *48* 559–569
Takagi, S F G A Wyse and T Yajima, Anion permeability of the olfactory receptive membrane *J gen Physiol* 1966, *50* 473–489
Takagi, S F G A Wyse H Kitamura and K. Ito, The roles of sodium and potassium ions in the generation of the electro-olfactogram. *J gen. Physiol.* 1968 *51* 552–578.
Takagi, S F H. Kitamura, K. Imai and H. Takeuchi, Further studies on the roles of sodium and potassium in the generation of the electro-olfactogram *J gen. Physiol.* 1969 *53*. 115–130.
Tomita, T Electrical activity of vertebrate photoreceptors. *Quart Rev Biophys.* 1970 *3*. 179–222.

Most successful are the striking experiments of Getchell and Gesteland (1972). They showed that the response to odours, as measured by the EOG was destroyed when the olfactory epithelium was treated with the protein reagent, *n*-ethyl maleimide, except the response to odours that were present in high concentration during the treatment. These results strongly indicate that proteins are involved in the specific processes of olfaction. They do not, however prove that the primary interaction of the odorants is with protein If the odorants interact primarily with the lipid part of a lipo-protein complex, this may protect the protein part against the reagent. It is known that agents interacting with the lipid part of lipo-proteins may alter the reactivity of the protein part with protein reagents (Godin and Wan Ng 1973).

Thus, the model of olfactory transduction occurring primarily with the lipids in a lipid protein double coacervate seems consistent with these experiments.

The ability of the sense of smell to distinguish between enantiomers, best established in the case of carvone, is often regarded as evidence for odorant interaction with proteins. However phospholipids also show enantiomerism (the β-carbon in the glycerol residue is asymmetrical) so there is in principle no reason why lipid systems could not have the same ability A rigid receptor site, such as may occur in proteins, would be expected to give a better discrimination but Friedman and Miller (1971) conclude that carvone probably does not interact with such a site Also the results of Leiterg *et al* (1971) suggest that the carvone enantiomers are not drastically different physiologically as the experimental subjects confused them in about 20% of the cases.

Conclusions

It seems that the olfactory receptor membrane may well contain regions where its lipids are organized essentially as in lipid–protein coacervates. This model can in principle explain much of the available physiological data. In particular it explains how odorants can cause permeability changes in the membrane and how different receptor cells can have different sensitivity patterns towards odorants. Both physicochemical data about the model and physiological data about the cells are insufficient to allow a more detailed evaluation of the model As further experimental work is possible along both lines, there is hope for future improvement.

References

ASH, K. O Ascorbic Acid cofactor I rabbit lfactory preparations. *Science* 1969 *165* 901–90..

BLANK, D L Mechanism underlying the nalysis of odorant quality t the level of the olfactory mucosa

II. Receptor selective sensitivity *Ann N Y Acad Sci.* 1974 *237* 91 101

BOECKH, J K. E. KAISSLING and D SCHNEIDER Insect lfactory receptors. *Cold Spr Harb S mp. quant*

Biol. 1965 *30* 263–280.

BOOIJ H L., In *Colloid Science* Ed. H. R. Kruyt Elsevier 1949 715–718

BUNGENBERG DE JONG, H G and G G P SAUBERT Kolloidmodelle biologischer Vorgänge. *Protoplasma*

1937 *28* 329–344

BUNGENBERG DE JONG, H G., G G P SAUBERT and H L. BOOIJ Der Einfluss rganischer Nichtelektrolyte

auf Oleat und Phosphatid koazervat III *Protoplasma* 1938 *29* 481–497

CHERRY R. J G H. DODD and D CHAPMAN, Small molecule-lipid membrane interactions and the

puncturi g theory of olfaction. *Biochim biophys. Acta* (Amst) 1970. *211* 409–416.

DAVIES, J T Recent developments in the "penetration nd puncturing theory of odour I *Taste*

and Smell in Vertebrates Ed G E. W Wolstenholme nd J Knight, Church ll 1970. 65-281

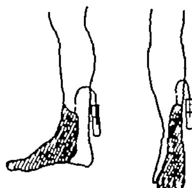


Fig. 1 Leg lymph cannulation. Hatched field indicates area drained by the cannulated lymph vessel. The tissues drained are skin, subcutaneous tissue and presumably muscular [illegible]

[illegible]on of cells, proteins and enzymes was determined, and its dependence on the body position, muscular activity and various procedures known to increase blood capillary filtration analyzed. The present paper deals with the variation in lymph flow and protein and enzyme concentration during night rest and normal daily activities.

Material and Methods

[illegible] healthy male volunteers aged 19 to 27 were studied. A superficial lymph vessel was cannulated under sterile conditions on both legs about 15 cm above the ankle, according to the technique previously described (Engeset et al. 1973). This lymph vessel drained skin, subcutaneous and perimuscular tissue of the foot of an area approximately as outlined in Fig. 1. The cannulation failed on one leg. Lymph was collected into sealed test tubes with or without 20 I.U. dry heparin—depending on the studies—and flow measured.

For protein and enzyme studies, lymph samples were spun down to remove cells. The supernatant was immediately stored at −20°C in sealed test tubes until it could be analyzed. Blood samples were taken from the cubital vein at the end of lymph sampling periods, and parallel studies carried out. Total protein concentration was determined by Biuret method, electrophoresis was performed on cellulose acetate strips. Lactate dehydrogenase (LDH) and alkaline phosphatase (AP) activity were determined with the Scandinavian recommended methods (see reference) using the LKB reaction rate analyzer. Lymph protein and enzyme output and lymph serum concentrations were calculated. Because of significant variations in basic parameters between the individuals and between legs of the same individual, due most also to different topography and caliber of the cannulated vessels, and because from day to day variation on the same leg the results have been presented as percentage of the night value from the same leg over the same 24 h period. The range of protein and enzyme concentration and lymph flow has also been presented in absolute numbers.

Experimental schedule

Studies were started on the third day after cannulation. The 24 h period was divided into 9 intervals of different duration devoted to various activities.

- 23.30–06.30 Night rest.
- 06.30–08.00 Getting up, morning activities (morning toilet, breakfast and journey to the hospital).
- 08.00–09.00 Lying in the horizontal position.
- 09.00–12.00 Fast [illegible]
- 12.00–13.00 Lying in the horizontal position.
- 13.00–15.00 Various procedures increasing capillary filtration.
- 15.00–16.00 Lying in the horizontal position.
- 16.00–19.00 Ordinary afternoon activities.
- 19.00–23.30 Ordinary evening activities.

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Twenty Four Hour Variation in Flow and Composition of Leg Lymph in Normal Men

By

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Abstract

ENGESET A. W OLSZEWSKI, P M JÆGER, J SOKOLOWSKI and L. THEODORSEN. *T[illegible] four hour variation in flow and composition of leg lymph in normal men* Acta phys[illegible] scand. 1977 99 140–148

The twenty-four hou variation in concentration and output f total protein, lactat dehydrogenase [illegible] alkaline phosphatase has been tudied i the peripheral lymph from the legs of 5 healthy volunteers [illegible] period of fi d ys. The highest concentration of these proteins was found in lymph collected during [illegible] first two hours after night s rest. During the day a continuous decrease in concentration occurred [illegible] [illegible] y direct correlat to lymph flow The variation in lymph protein concentration between early mor[illegible] and late day was about 40 [illegible], lactate dehydrogenase and alkaline phosphatase bout 90 and 45 [illegible] tively

K y ords Activity cycles, body fluids, capillary permeability circadia rhythm, enzymes, extracell[illegible] fluid lymph, lymphedema, proteins

The flow rate and composition of peripheral lymph depend *inter alia* on the hydrostatic [illegible] oncotic pressures in the intravascular and extravascular compartments (Starling 18[illegible] Drinker and Field 1933 Guyton 1963 Courtice 1971), regional permeability of blood cap[illegible] laries (Mayerson *et al* 1960) and initial lymphatics (Casley-Smith 1970), and on molecu[illegible] weight and size of the various protein components in blood and tissue fluid (Pappenheim[illegible] *et al* 1951 Garlick and Renkin 1970 Grotte 1956, Renkin 1964) Variations in lymph fl[illegible] and composition will occur depending on the functional status and position of the tis[illegible] from where lymph is collected Preliminary studies of the 24 h variation of leg lymph fl[illegible] and composition in patients with malignancies revealed a pattern of variation which to so[illegible] extent was in contradiction to that found in animal experiments (Jæger 1975) The findin[illegible] were confirmed in the present study of peripheral lymph in healthy male volunteers, follow[illegible] systematically throughout several days. The 24 h variation in the lym h flow concentr[illegible]

. I. *Total protein concentration and enzyme activity in leg lymph from 5 healthy volunteers. Mean of 5 days for each leg and for all men ± S.E.*

Leg	Total protein g/100 ml		Alkaline phosphatase U/l		Lactate dehydrogenase U/l	
	Night	24-hr period	Night	24-hr period	Night	24-hr period
L	1.70	1.80	16	11	42	57
R	2.18	1.97	26	18	82	63
R	1.64	1.42	20	15	53	36
L	2.03	1.85	30	20	48	33
R	2.69	1.80	25	18	63	45
L	2.43	2.14	33	26	92	57
R	2.40	1.99	35	26	42	26
L	2.20	2.11	36	28	74	56
R	2.78	2.46	48	37	95	72
m	2.16	1.97	29.9	22.9	65.07	49.22
	0.12	0.083	3.19	2.32	6.96	5.13

mph protein concentration and output

mph total protein concentration was in the range of 1.37 to 2.98 g/100 ml. The mean ht concentration was 2.14±0.1 (1 S.E.) g/100 ml. The concentration varied between lividuals and between the two legs of the same man. All legs showed the same pattern of riation throughout the 24 h period. There was an increase of about 20 % after getting up the morning, then a steady drop towards the end of the day to values below the night level able I, Fig. 2). No direct correlation between the flow and lymph protein concentration as found throughout the day (Fig. 3). Increase in flow after assuming an upright position the morning was accompanied by the highest increase in protein concentration. Then

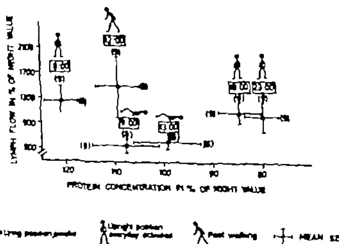


Fig. 3. 24 hour relationship between lymph flow and lymph protein concentration, both expressed as per cent of the night value. Mean values from 9 legs for 5 days ±1 S.E.

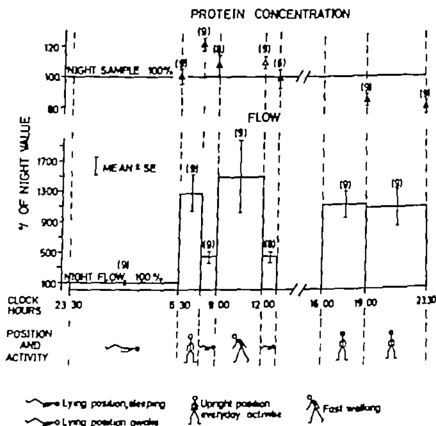


Fig. 2. 24-hour variation in leg lymph protein concentration and lymph flow expressed as % of night values from the same leg over the same 24-hour period Mean values from 9 legs for 5 days ± one standard error Symbols indicat the positio and activity during lymph sampling. Lymph collected between 13.00 and 16.00 is excluded because of special experiments during this period.

Lymph was collected continuously into test tubes replaced at the end of each sampling period, listed above. The content of each tube, comprising all lymph sampled during the preceding interval, was analyzed separately Lymph volume and protein concentration were determined in all samples. The enzyme concentration was not analyzed in the samples collected after the 60-minute periods in the horizontal position because the samples were too small.

Blood samples were taken at the following times 08.00, 09 00, 12.00, 13 00 and 16.00 hours.

Results

Lymph flow

The flow was in the range of 0 01 to 0.55 ml/h throughout the observation period. There were major individual variations and also variation between the two legs of the same man as well as from day to day variations. The mean night flow was 0.24 ± 0 056 (1 S E.) ml/h. There was a mean 13-fold increase in flow after getting up in the morning (Fig. 2). The flow dropped during the 60-minute periods in the horizontal position, but the mean was still almost 5 times higher than during night rest. Fast walking increased the flow to about 15 times the night values. During the evening the flow was about 10 times higher than the night flow

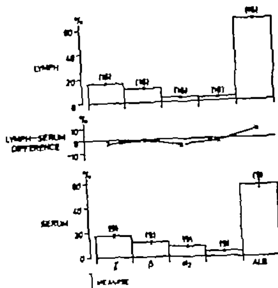


Fig. 3. Leg lymph and serum electrophoretic pattern. Mean values from 5 ... ±1 S.E. (9 legs examined). The Student' t test used to compare the differences. Samples taken after 3 h of fast walking

Discussion

The main finding has been a marked 24-hour variation in lymph protein and enzyme concentration and output without any direct correlation between lymph flow and protein concentration. This differs with what has been observed in the peripheral lymph of running dogs (White, Field and Drinker 1933, Garlick and Renkin 1970, Joyner *et al.* 1973, Saas 1964). They found according to Drinker's theory (1933) on lymph formation, that the protein concentration was inversely related to lymph flow when capillary permeability was constant. In the present study there was always a low lymph flow during the night, and the protein and enzyme concentration was relatively high. After getting up in the morning, both lymph flow and concentration increased significantly. During subsequent fast walking the protein concentration was maintained at a relatively high level regardless of a high lymph flow. Finally, lymph protein and enzyme concentration and lymph to serum ratio revealed a tendency to decrease in the course of the day. We consider the following mechanisms most likely responsible for the observed variations in lymph flow and composition.

Night lymph

In the horizontal position during sleep there is a low capillary hydrostatic pressure (Landis 1930) with decreased filtration and may be also increased reabsorption of excess water and solutes from the tissue fluid. Furthermore, there is a reduced muscular activity which reduces the propulsion of lymph in the collecting trunks (Taylor *et al.* 1973). For this reason the lymph flow during the night will be low.

There is, however, all the time a diffusion of proteins and other large molecules throughout the walls of the exchange vessels (Garlick and Renkin 1970). All this will result in a high protein concentration of the interstitial fluid. The lymph sampled in the vials throughout the night will, however, be a mixture of

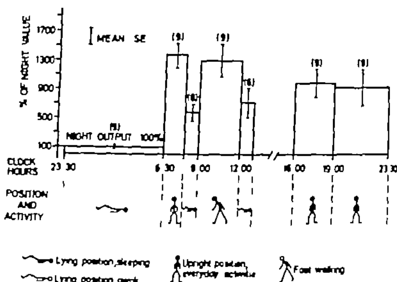


Fig. 4 24-hour variation in leg lymph p otein output expressed as % of night value from the same the same 24 h period. Mea values from 9 legs for 5 days ± 1 S.E.

during fast walking, protein concentration remained high despite a 15-fold incre lymph flow

Lymph protein output per hour reached the highest values in the first morning samp a result of an abundant flow of concentrated lymph (Fig. 4) During fast walking it wa an average 13 times higher than during the night During rest periods it decreased to a half the walking values.

Serum protein concentration was not studied early in the morning (before getting and during the evening. During the day the serum protein concentration dropped by a 5% following 60 min of rest in the horizontal position, and increased slightly after wal (Fig. 6)

Electrophoresis (of samples obtained after 3 hours of fast walking) revealed a 7 higher albumin and a 4 0% lower alpha 2-globulin concentration in lymph than in se (Fig. 5)

Enzymes

The enzyme concentration followed the protein pattern during the observation pe (Fig. 6) The range of AP activity was 12 to 53 U/l The mean night AP concentratio lymph was 31 7 ± 3 8 (1 S E.) U/l The first morning lymph sample had 20% higher va than the night sample from then on a steady decrease was observed. The LDH hy activity was within a range of 5 to 157 U/l The mean night value of LDH in lymph 79 8 ± 9 8 (1 S.E.) U/l It was 52% higher in the first morning sample than during the ni It decreased gradually towards the end of the day down to 50% of the night value in last evening sample (Table I Fig. 6)

Serum LDH and AP concentration revealed the same type of fluctuation as the t serum protein value with an average of 5 . fall during rest in the horizontal position (Fig A decrease in the ratio lymph/serum of LDH and AP was observed

face may take place due to opening of capillaries, closed at low pressure, and stretching capillaries which may evok "stretch pore effect" (Shirley *et al* 1957) increasing lymph mation.

mph collected during fast walking

e washout of proteins accumulated during the night is probably a time-consuming process it may explain why a high-protein concentration was still maintained during fast walking the morning, despite a high lymph flow In addition, increased capillary filtration of otein in active tissue may play some role.

mph collected during short rest periods in the horizontal position

e relatively high mean lymph flow and lymph protein concentration during the rest riod between 8–9 and 12 13 hours compared with the night period was most likely caused the postexercise hyperemia following muscular activity with most capillaries open, thus gh filtration area.

mph sampled towards the end of the day

he mechanism behind the continuous decrease in protein concentration of lymph during e day despite constant or somewhat reduced lymph flow is difficult to explain. It might be at the ashing out of the proteins accumulated in the tissue space during the night takes long time that it will influence the protein concentration of lymph produced in the evening.

Electrophoresis

higher concentration of albumin and a lower concentration of alpha-2-globulin were ound in lymph compared with serum. The alpha-2-macroglobulin accounts for the bulk f the alpha -band in the serum. This has a high molecular weight. The finding is therefore n accordance with the molecular sieving theory

nzymes

o different enzymes were chosen for parallel studies. These were lactate dehydrogenase hich can be locally produced by the drained tissue, and alkaline phosphatase originating other tissues and easily equilibrated in body fluids. The variation of concentration of nzymes in the serum followed that of the protein, with a fall during rest (Fig. 6). The lymph concentration of AP followed the same pattern as lymph concentration of proteins. The concentration of LDH is somewhat different, the curve is steeper and there is a steady fall until the last sample taken late in the evening. The reason for the continuous drop in LDH throughout the evening might be that the amount of locally-produced LDH (muscle enzymes) decreases with reduced muscle activity

To conclude, a 24 h variation in concentration of protein and enzymes in periphera lymph has been found. The mechanism behind this is discussed without taking into consider ation parameters such as diurnal variation in cell metabolism, hormone status and capillar filtration. Before a more sophisticated discussion can be fruitful, these parameters hav t be studied and also the direct effect of the erect and supine position. Such experiments ar

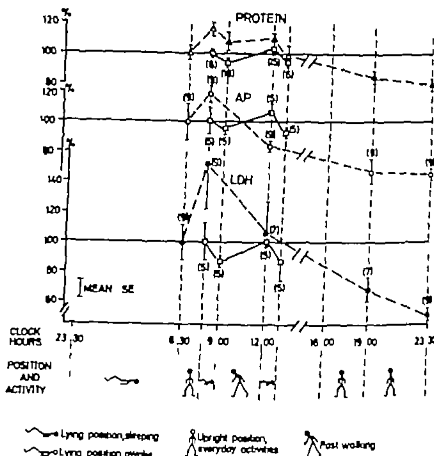


Fig. 6. 24-hour variatio In leg lymph — and serum —— protein concentration, alk line and lact dehydrogenase activity Lymph values expressed as of night sample from the same leg over the 24-hour period. Serum values expressed as f the first morning sample on the same day Mean values from 9 legs fo 5 days ± 1 S.E.

a) low-protein concentration fluid produced before bedtime accumulated in interstitial tissue space, lymphatics and catheter (the last one containing about 0 1 ml)

b) lymph produced in bed prior to sleep while the volunteer might still be moving and before blood pressure and filtration have reached the low sleeping values

c) lymph produced during sleep as described

Because of this mixture the protein concentration in the night lymph will always be somewhat lower than the protein concentration of lymph produced during sleep

Early morning lymph

Because of markedly reduced lymph propulsion during sleep concentrated fluid will accumulate in the tissue lymph capillaries, lymph vessels and catheter Assuming the upright position in the morning several mechanisms become effective which bring about squeezing and washing out this preformed fluid. These are an increase of venous pressure (Asmussen 1943 Grill 1937 Henry *et al.* 1955) and expansion of capacitance vessels (Gauger and Sieker 1962) which elevate the tissue pressure (Mayerson and Burch 1940) and muscular movements which promote lymph flow (Taylor *et al* 1973). Some increa capillary filtration

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Flow and Composition of Leg Lymph in Normal Men during Venous Stasis, Muscular Activity and Local Hyperthermia

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Abstract

…ZEWSKI, W., A. ENGESET, P. M. JAEGER, J. SOKOLOWSKI and L. THEODORSEN. *Flow and composition of leg lymph in normal men during venous stasis, muscular activity and local hyperthermia.* Acta physiol. scand. 1977. 99. 149–155.

…nges in normal human leg lymph protein concentration, output, and lymph flow/lymph protein con…tration relationship, as well as lactate dehydrogenase and alkaline phosphatase activity were followed …ing procedures known to increase capillary filtration as venous stasis, muscular exercise and warming …tissues. Lymph flow increased by 83 % during two hour ergometer cycling, and by 117 % during two … warm water foot bath. During … two hour period of venous stasis lymph flow dropped by 50 … …ere … as an increase in lymph flow during the rest period following all three types of experiment, most …nounced after foot warming. An inverse relationship between the lymph flow rate and lymph protein …centration … is found. Lymph enzymes followed the same pattern of changes as total protein.

…y words: Activity cycles, body fluids, capillary permeability, circadian rhythm, enzymes, extracellular …id, lymph, lymphedema, proteins

…he rate of formation, flow and composition of lymph in human leg depends mainly on the …osition of the extremity, its muscular activity and ambient temperature. Change of posi…ion from the horizontal to the upright is accompanied by a rise in venous hydrostatic …ressure (Henry *et al.* 1955). Walking causes an increase in blood flow through the leg tissues …nd muscle pump promotes lymph flow (Taylor *et al.* 1973). Ambient temperature may …nfluence lymph formation by dilatation or constriction of skin microcirculation (Henry *et al.* 1955). All the three described physical factors may, to different degrees, affect lymph volume, protein concentration and lymph/serum protein ration. The knowledge of these differences would be important for understanding of the mechanism of interstitial fluid …volume regulation in human leg.

in progress. A practical consequence of the observed 24 h variation is that data on periphe lymph flow and composition to be informative must always be correlated with the sam conditions.

References

ASMUSSEN, E. The distribution of the blood between the lower extremities and the rest of the body *Acta physiol scand* 1943 *5* 31–41

CASLEY SMITH J R., The functioning of the lymphatic system under normal and pathological cond *Progress in Lymphology* Ed. F Ruttiman, Thieme, Stuttgart, 1970.

Committee of Enzymes of the Scandinavian Soc. for Clinical Chemistry and Clinical Physiology Recommended methods for the determination of 4 enzymes in blood. *Scand. J clin. Lab. Invest.* 1974 291–306.

COURTICE, E. C. Lymph and plasma proteins Barriers to their mo ement throughout the extracell fluid. *Lymphology* 1971 *1* 9–11

DRINKER, C. K. and M E. FIELD, *Lymphatics, lymph, and tissue fluid.* Williams and Wilkins, Baltimore 1933

ENGESET A B. HAGER, A NESHEIM, and A. KOLBENSTVEDT Studies on human peripheral lymph. I. *Lymphology* 1973 *6* 1 5

GARLICK D G and E. M RENKIN, Transport of large molecules from plasma to interstitial fluid r lymph in dogs. *Amer J Physiol.* 1970. *19* 1595–1605.

GAUGER, O H. and H O SIEKER, Properties of eins i vivo Integrated effects of their smooth muscle *Physiol Rev* 1962. *42*, Suppl 5 283.

GRILL, C., Investigations with displacements in blood mass due to changes in body positions *Acta m scand* 1937 *92* 267 277

GROTTE, G., Passage of dextran molecules across the blood-lymph barrier *Acta chir scand.* 1956 Suppl 211

GUYTON, A C., A concept of negative interstitial fluid pressure based on pressures in implanted perforated capsules. *Circulat Res* 1963 *12*. 399–414

GUYTON, A. C. and T G COLEMAN, Regulation of interstitial fluid volume a d pressure *Ann. N Y Acad Sci* 1968 *150* 537 547

HEN Y J P SLAUGHTER, O L. and GREINER, T A medical massage suit for a continuous car *Angiology* 1955 *6* 482–490.

JÆGER, P M *Human peripheral lymph.* Thesis. Subm tted t Uni ersity of Oslo in particular fulfilment of the requirement for the degree of C nd pharm. (In Norwegian). 1975

JOYNER, L. L. R D CARTER nd E M RENKIN, I fluence of lymph flow rate on concentrations of protein and dextran in dog leg lymph. *Lymphology* 1973 *6* 181 186

LANDIS, E. M Micro-injection studies f capillary blood pressure in huma skin *Heart* 1930. *15*. 209

MAYERSON, H. S. and G E. BURCH, Relationship of tissue (subcutaneous and i tramuscula) and venous pressures to syncope induced in ma by gravity *Amer J Ph siol* 1940. *128* 258–69

MAYERSON, H S C. G WOLFRAM, H. W SHIRLEY and K. WASSERMAN, Regional d fferences in capillary permeability *Amer J Physiol* 1960. *198* 155–162.

PAPPENHEIMER, J R E. M RENKIN and L. M BORRERO, Filtration, d ffusion nd molecular sieving through peripheral capillary membranes. A contribution to the pore theory of capillary permeability *Amer J Physiol.* 1951 *167* 13–46.

RENKIN, E. M Transport of large molecules across capillary w lls. *Physiologist* 1964 *7* 13–28.

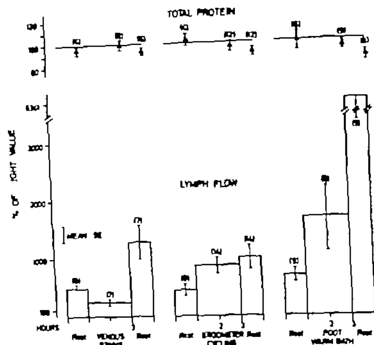
SASS, M B. *Lymphatic system of the reproduct ve organs in pregnancy* Ph.D Thesis in Australian National University Canberra. 1964

SHIRLEY H H., C. G WOLFRAM, K. WASSERMAN and H S. M YERSON, Capillary permeabil ty to macromolecules. stretch pore phenomenon *Amer J Physiol* 1957 *190* 189–193

STARLING, E. H Production nd bsorption of lymph I *T xtbook of Ph siology* Ed E. A Schäfer MacMillan. New York. 1898. I

TAYLOR, A., H GIBSON, H J GRANGER and A. C. GUYTON, The interaction between intracapillary and tissue forces in the overall regulation of i ters tial fluid olume *L mphology* 1973. *6*. 192 208.

WHITE J C., M E. FIELD nd C. K DRINKER, On the protein content and normal flow of lymph from the foot of the d g. *Amer J Physiol.* 1933 *103* 34–44.



ig. 1 Lymph flow and protein concentration in the leg before, during, and after leg venous stasis, ergometer cycling, and warm water bath. Values from 5 min expressed in percentage as means of night values in the same leg over the same 24 h period ± 1 S.E.

Fig. 3) Most pronounced changes were seen in the rest period after warm water bath. Lymph flow increased by 273% reaching values 63 times higher than the night samples. Protein concentration fell by 9.3%. Protein output was doubled and 33 times higher than the night value. LDH and AP dropped by 23.4% and 15.5% respectively. The increase in flow was accompanied by decrease in protein concentration (Fig. 4).

Discussion

The purpose of the present study was to follow the protein and enzyme transport to the interstitial fluid and lymph in three experimental situations, to some extent different in the mechanism of increase in capillary filtration. In the first, venous hydrostatic pressure was increased in leg using sphygmomanometer cuff. This elevated the capillary filtration pressure. In the second, blood flow in the leg was stimulated by strenuous ergometer cycling. This opened muscle and also skin capillaries and increased capillary filtration surface area. In the third, leg tissues were warmed to 41°C, which produced maximum opening of skin capillaries and their dilatation with subsequent increase of filtration area and enhanced permeability of the capillary wall (Garlick and Renkin 1970, Arthurson *et al.* 1969, Courtice and Sabine 1966).

A decrease of lymph flow was found during venous stasis, a slight increase during ergo-

In the present study changes in leg lymph protein concentration and output, lymph flow-protein concentration relationship and lactate dehydrogenase (LDH) and alkaline phosphatase (AP) activity were observed during leg venous stasis, ergometer cycling and warming of the foot.

Materials and Methods

The present investigations were carried out on the same group of 5 young men as in the previous study (Engeset *et al* 1976). Each had a leg superficial lymphatic cannulated and lymph collected. Details of lymph collection method were described earlier (Engeset *et al.* 1973). The collected lymph was derived from the skin, subcutaneous tissue and muscular fascia of the anterior and medial aspects of foot and lower part of the leg. Investigations were carried out between 1 p.m. and 3 p m preceded and followed by periods of rest in the horizontal position Only one type of experiment was performed on each individual each day. In group 1 a sphygmomanometer cuff was placed on the thigh and inflated to a pressure of 30 mm Hg. In group 2, ergometer cycling was performed at a speed of 30 km/h with 2 kg load. In group 3, feet were immersed in a water bath with a constant temperature of 41°C. Lymph was collected continuously into test tubes replaced at the end of each experimental and rest period. The content of each tube, containing all lymph sampled during the preceding interval, was analyzed separately. Lymph flow, lymph total protein concentration and output, LDH and AP activity were measured (as previously described by Engeset *et al* 1976). In the available blood samples the same protein and enzyme studies were performed. Because of the significant differences in normal values of lymph flow, protein concentration and enzyme activity between the individuals and even legs of the same individual, and also from day to day variation (Engeset *et al* 1976), the results were presented in percent of the night values calculated for each leg every 4 h.

Results

Venous stasis

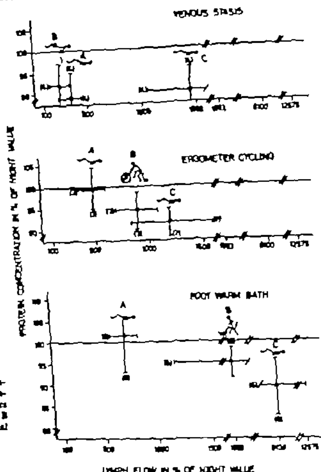
During leg venous stasis, lymph flow decreased by 50% compared with the rest period, and protein concentration increased by 4% (Fig. 1). Protein output fell by around 50% (Fig. 2). LDH and AP remained about the same level as at the beginning of the rest period (Fig. 3). Following release of the venous cuff lymph flow increased by 430%, at the same time protein concentration, LDH and AP activity decreased by 6%, 25%, and 12% respectively. Protein output rose by 416% compared with the venous stasis period. Fig. 4 points to an inverse relationship between lymph flow and lymph protein concentration.

Ergometer cycling

Ergometer cycling increased lymph flow by 83% compared with the preceding rest period (Fig. 1). Protein concentration fell by 4%. Protein output was 52% higher than during the rest period (Fig. 2). LDH decreased slightly and AP remained unchanged when compared with the estimates from the beginning of the rest period (Fig. 3). There was a 15% increase in lymph flow in the period immediately after exercise and a decrease in protein concentration, LDH and AP by 5%, 3% and 9% respectively. There was a trend towards a decrease in lymph protein concentration with increase in lymph flow (Fig. 4).

Warm water bath

Warm water foot bath (41 C) brought about 117% increase in lymph flow (Fig. 1). Protein concentration dropped by 5% (Fig. 1). Protein output increased by 104% (Fig. 2). LDH and AP dropped by 28% and 17% when compared with the beginning of the rest period



g. 4 Lymph total protein concentration to lymph flow relationship during the same procedures shown in Fig. 1 A before, B during and C after experimental procedure

xperiments performed on dogs (Szabo *et al.* 1963). The mechanism of this observation emains unclear In the period of venous stasis the extremity was immobilized in order to nactivate the muscle pump as much as possible. The main force promoting lymph propulsion was then absent. In this situation capillary filtrate probably first of all filled the interstitial space, which caused a temporary drop in lymph flow This may be in agreement with the observation that elevation of the venous pressure is followed by an increase in lymph space (Garlick and Renkin 1970) Evacuation of the excess interstitial fluid began when the local tissue space pressure/volume values exceeded the safety limits (Guyton *et al* 1971), which in the present study probably coincided with the release of the venous cuff

During muscular exercise lymph flow was twice as high as during the preceding rest period. For interpretation of this observation one should remember that tissues under investigation were skin, subcutaneous tissue and muscular fascia. Only a small fraction of collected lymph could originat from muscles. Two factors should be considered as responsible for the increase in lymph flow The first is the "muscular pump" which provides most of the driving force for the lymph flow in the limb superficial lymphatic system. The second

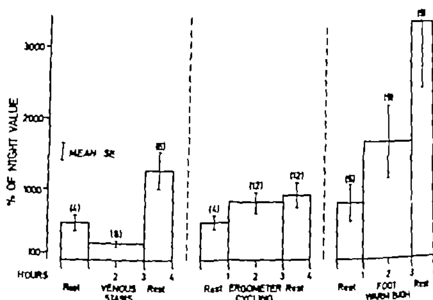


Fig. 2. Protein output in leg lymph during the same procedures as shown in Fig. 1

meter cycling, and an evident rise during warming of the foot After completion of the experiment a high lymph flow was noted in all three groups. An inverse relationship between lymph flow and protein concentration was observed.

During venous obstruction a decrease in lymph flow in all individuals was observed despite evident clinical signs of venous stasis. The same finding was described by others

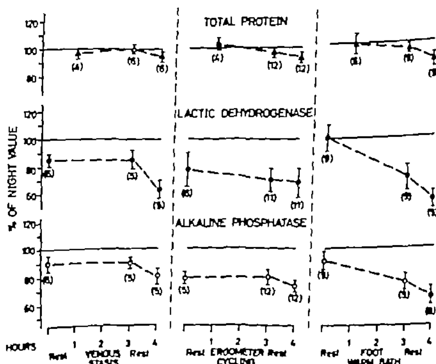


Fig. 3 Lactate dehydrogenase alkaline phosphatase activity and total protein concentration during same procedures as shown in Fig. 1

References

...son, G N.-H. Arfors, K. Arturson, G. Grotte and P. Malmberg, The transport of macromolecules across the blood-lymph barrier. *5th Europ. Conf. Microcirculation*, Gothenburg, 1968. No. 10. ...–33. Karger Basel/New York, 1969.

...tice, F. C. and M. S. Sarwar, The effect of changes in local temperature on the transfer of proteins and lipoproteins from plasma to lymph in the normal and injured paw of the hypercholesterolemic rabbit. *... J. exp. Biol. med. Sci.* 1966. *44* ...3–36.

...ert A. B. Hauge, A. Nicolaysen and A. Kolbenstvedt. Studies on human peripheral lymph. *Lymphology* 1973. *6*. 1–5.

...ert, A., Olszewski, P. M. Jaeger, J. Sokolowski and L. Theodorsen, Twenty-four hour variation in flow and composition of leg lymph in normal men. *Acta physiol. scand.* 1977. *99*. 140–148.

...ick, D. G. and E. M. Renkin, Transport of large molecules from plasma to interstitial fluid and lymph in dogs. *Amer. J. Physiol.* 1970. *219*. 1595–1605.

...ton, A. C. H. J. Granger and A. E. Taylor, Interstitial fluid pressure. *Physiol. Rev.* 1071. *51*. 527 ...3.

...ey J. P. O. L. Slaughter and T. Gurner, A medical message unit for continuous wear. *Angiology* ...55. *6*. 482–490.

...ng, E. M. Micro-injection studies of capillary blood pressure in human skin. *Heart* 1930. 209–228.

...on, E. M. and Pappenheimer, J. R. Exchange of substances through the capillary wall. In. *Handbook of Physiology* (W. F. Hamilton and P. Dow. Eds.) Section ... Vol. II, 961 1034. Williams and Wilkins, Baltimore, 1963.

...no, G. Ultrastructure of the vascular membrane. In. *Handbook of Physiology* (W. F. Hamilton and ... Dow. Eds.) Section 2. Circulation. Vol. III. Chapter 64. 2293–2375. Williams and Wilkins, Baltimore, ...963.

...zewski, W. Unpublished observations.

...ver, T. A. W. C. Hartner and J. F. Heath, Interstitial fluid pressure and vasomotor responses in ... *Amer. J. Physiol.* 1974. *226*. 353–356.

...n, M. B. *Lymphatic system of the reproductive organs in pregnancy.* Ph.D. thesis in Australian National University. Canberra, 1964.

...no, G. Z. Magyar and M. Papp. Correlation between capillary filtration and lymph flow in venous congestion. *Acta med. Acad. Sci. Hung.* 1963. *19*. 185–191.

...lor, A. H. Gibson, H. J. Granger and A. C. Guyton, The interaction between intracapillary and ... forces in the overall regulation of interstitial fluid volume. *Lymphology* 1973. *6*. 192–208.

...ite, J. C. M. E. Field and C. K. Drinker, On the protein content and normal flow from the foot of the dog. *Amer. J. Physiol.* 1933. *103*. 34–44.

is an increase in skin lymph formation due to hyperemia accompanying increased mu
blood flow It has been found that strong muscular exercise of the lower limb in ma
duces a 2–3-fold rise in skin blood flow (Olszewski 1963).

High lymph flow during warming of the skin may be explained by increased cap
filtration due to opening of more capillaries and their dilatation followed by a rise in ca
vascular hydrostatic pressure (Landis 1930 Reaves *et al* 1974). There is also an incre
permeability of the capillary wall to protein (Arthurson *et al* 1969 Courtice and Sa
1966, Garlick and Renkin 1970) which has been attributed to the opening of large gaps b
tween endothelial cells of postcapillary venules (Majno 1963) For high postwarming ly
flow a protracted effect of hyperthermia on the local blood flow regulation with vasodila
tion and increased permeability and accumulation of large amounts of interstitial fluid
tissues may be responsible

In all groups in the study an overall tendency although of a different degree, toward
decrease in protein concentration with increase in lymph flow was noted. This would
main in agreement with the findings of other authors (Arthurson *et al* 1969 Courtice a
Sabine 1966 Garlick and Renkin 1970, Landis and Pappenheimer 1963 Sass 1964 Sz
et al 1963 White *et al* 1933). *In the group with ergometer cycling a moderate rise in ly*
flow and decrease in protein concentration may be accounted for by increase in capil
surface filtration area No rise in capillary permeability would be expected.

In the group with venous obstruction, the proportionality between decrease in prot
concentration and increase in lymph flow would indicate enlargement of capillary filtrat
area In local hyperthermia, an extremely high rise in lymph flow was accompanied b
rather small decrease in protein concentration, compared with the two former groups
creased filtration area together with enhanced capillary permeability seem to be respon
for these findings.

In our previous study on 24 hour variation of leg lymph flow and composition, no r
tionship between lymph flow and protein concentration was found (Engeset *et al.* 19
We attributed this to the time required to wash protein-rich lymph formed during rest
fore periods of activity out of the interstitial space and lymph vessels, thus a lag betw
formation and collection of lymph. Especially accumulation of protein during the night
might have played a role here. The present experiments were performed in the aftern
when the proteinrich tissue fluid formed during the night has probably been washed
Furthermore, high capillary filtration which accompanied the three present types of pro
dure permitted fast filling of interstitial space and promoted lymph flow so that flow
protein concentration could more accurately reflect the rate of lymph formation in
tissues.

The lymph enzymes followed the same pattern of changes as did total protein. LDH
AP activity showed a tendency to decrease during all three types of experiment. This ef
was most evident during the period immediately after procedures increasing capillary fil
tion, especially after warm water bath

ds in skeletal muscle might contribute to a disturbance of the lipid metabolism dur
nol intake in intact animals and man.
ous studies on acetate metabolism in skeletal muscle have included investigation of
ct of acetate on the metabolism of exogenous glucose (Karlsson *et al.* 1976). The
report deals with the effect of acetate on the metabolism of palmitate a long-chain
id, in the perfused hind-quarter of the rat.

Material and methods

Animals

Sprague-Dawley rats weighing 200–240 g, purchased from Anticimex, Sollentuna, Sweden were
he rats were fed *ad libitum* on standard small-animal diet obtained from Astra-Ewos, Södertälje,
, and were allowed free access to water prior to the experiments.

Chemicals

d analytical grade laboratory reagents were obtained from E. Merck AG, Darmstadt, West Ger
Enzymes and co-enzymes were obtained from Boehringer Mannheim, West Germany
a Chemical Co St. Louis, Miss USA. Solutions of hexobarbital (100 mg/ml) ere freshly pre-
from hexobarbital powder (Evipan -sodium, Bayer AG, Leverkusen, West Germany). Bovine serum
n (fraction V) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, England. Stock
ns of albumin (10) were prepared in Krebs–Henseleit high-bicarbonate buffer (Krebs and Henseleit
and dialyzed at 4°C against four-fold volume of the same buffer for 3 24 h.
^{14}C-acetate (58 5 mCi/mmol) and 1-^{14}C-palmitic acid (57 9 mCi/mmol) were purchased from the
chemical Centre, Amersham, England. Chemicals for measurement of radioactivity were obtained
Packard Instr Co Inc., Ill USA and Koch-Light Laboratories Ltd Buckinghamshire, England.

Perfusion technique and experimental procedure

method of rat hind-quarter perfusion used was that described by Ruderman *et al* (1971) All perfu-
were done on skinned animals with the hind-legs at rest. The perfusion medium (170 ml) as the same
viously used at our laboratory (Karlsson *et al.* 1975) with an initial concentration of lactate of 1 9
Glucose consumption and lactate production due to glycolysis in the bovine red cells during the
imental period was negligible.
e rats were anaesthetized by intra-peritoneal injections of hexobarbital (25 mg/100 g b wt.). The first
l of the perfusate was discarded and the medium was then recycled Substrates to be studied were
d at 10 min. Stock solutions of palmitate (33 mM) were freshly prepared by mixing 0 5 mmol of pal-
c acid and 15 ml of water These solutions were heated to 70°C for 15 min. Complete solution of the
acid occurred after drop-wise addition of 1 M NaOH The quantity of fatty acid solution to be added
he medium was mixed while still warm with pre-warmed dialyzed 25 bovine albumin (prepared as the
solution) and this solution was added to the medium as single dose Acetate (1.3 M stock solution
ater) was added as single doses followed by continuous infusion of acetate into the medium at rate of
mol h g muscle tissue as described previously (Karlsson *et al* 1975) Samples of the medium for ana
s of palmitate were taken at 15, 25, 40, 55 and 70 min. Samples for the analysis of acetate, lactate and
uvate were usually taken at 15, 40 and 70 min Metabolic rates (µmol/h g wet weight) were calculated
m changes in substrate concentration in the perfusate during the experimental period.

Analytical methods

amples of the medium for analyses of the concentrations of lactate and pyruvate were added to 0 6 M
ClO The extracts ere neutralized with KOH and the precipitated $KClO_4$ was removed by centrifuga
n. The concentrations of lactate and pyruvate were measured in these extracts as described by Hohorst
al. (1959) Acetate and palmitate were determined in plasma according to Bergmeyer and Möllering
970) and Itaya and Ui (1965) respectively A freshly prepared standard solution of acetate (2 mM) in
bumin (4) was always run parallel to the plasma samples, when acetate was determined. Determina-
on of the oxygen consumption by the perfused tissue was done as previously described (Karlsson *et al.*
975). Oxygen measurements were usually done at 25, 35, 50 and 60 min.

Acta physiol. scand. 1977 99 156–165

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Influence of Acetate on the Metabolism of Palmitate in the Perfused Hind Quarter of the Rat

By

N Karlsson E. Fellenius and K. H Kiessling

Received 18 June 1976

Abstract

Karlsson, N., E. Fellenius and K H Kiessling *Influence of acetate on the meta of palmitate in the perfused hind-quarter of the rat* Acta physiol scand 1977 99 15

The metabolism of 1 ^{14}C-palmitate and its metabolic interaction with U-^{14}C-acetate ere studied perfused hind-quarter of the rat. 9 % of 1 ^{14}C palmitate taken up was oxidized to $^{14}CO_2$ account 7 of total oxygen consumption by the perfused tissue. Most label from 1 ^{14}C-palmitate as foun lipid fraction of the muscle tissue. I spite of a 40 % inh bition of palmitate oxidation, acetate only minor changes in the overall met bolism f palmitate. U ^{14}C-acetate was mainly oxidized to $^{14}CO_2$ oxygen consumption due to oxidation of acetate accounted for 20–30 % of the total xygen uptake amounts of ^{14}C-acetate were found in muscle l pids. The addition of palmitate did not alter the meta of acetate. It is concluded that the presence of palmitate did not affect ^{14}C-acetate metabolism. presence of acetate inh bited ^{14}C palmitate oxidation. The possible sites of interaction are discuss found interaction will probably not contribute t any major extent to the disturbed lipid metabolism in animals and ma during ethanol intake. No major changes in the tissue content of high-energy pho compounds were found in the presence of palmitate or cetat or both

Key words: Acetate metabolism, palmitate metabolism, skeletal muscle, muscle perfusion, ethan sumption

Acute and chronic administration of ethanol to man and animals is generally follow metabolic alterations in a number of organs and tissues (Wallgren and Barry III 1970, and Mardones 1971 Kissin and Begleiter 1971) One characteristic alteration caus ethanol is a disturbed lipid metabolism related to effects on adipose tissue or liver fu (Lieber 1974) Since the main metabolite of ethanol metabolism in the liver is acetate (quist 1962) a short-chain fatty acid, it is of interest to study how this metabolite in ad to a direct effect of ethanol contributes to the disturbance of the lipid metabolism S have shown that skeletal muscle is of quantitative importance for the elimination of a from the blood (Karlsson *et al* 1975) and long-chain fatty acids are known to be impo substrates for skeletal muscle (Keul *et al* 1972) Thus, an altered combustion of long-

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.t II The effect of acetate on the metabolic fate of 1-^{14}C-palmitate in the perfused hind-quarter of the rat

strate to ·lard ium	Palmitate uptake (μmol h g wet weight)	Incorporation of label into		Oxygen consumption due to oxidation of ^{14}C-palmitate to $^{14}CO_2$ ( )[a]	Recovery of added label ( )
		$^{14}CO_2$	Water soluble metabolites in muscle tissue		
		(μmol of palmitate/h g wet weight)			
palmitate, μM	0 94±0 06 (6)	0 087±0 008 (6)	0 066±0 010 (6)	7.3±0 8 (6)	95±1
palmitate, μM _tate, 2 mM acetate	0 90±0 05 (6)	0 062[b]±0 007 (6)	0 032[b]+0 003 (6)	5 6±0 8 (6)	93±

sults are expressed as mean S E ith the number of observations in parentheses. Experimental conditions: see Table I and Methods.
[a] orrection has not been made for re-oxidation of NADH by lactate dehydrogenase.
[b] gnificantly different from perfusion ithout acetate, p 0 05.

The oxygen consumption was not altered by the addition of acetate or palmitate or both able I) When the supply of exogenous substrates was restricted, as in the control ex eriment, the lactate concentration in the medium was essentially unchanged during the rfusion, but the addition of acetate or palmitat or both resulted in a net production lactate (Table I).

fluence of acetate on 1 ^{14}C-palmitate metabolism

bout 9 of total ^{14}C-palmitate taken up was oxidized to CO and about 7% was recovered water soluble metabolites in the muscle tissue (Table II). Most label taken up (about)%) as found in the chloroform extract of the muscle tissue. The addition of acetate aused a statistically significant decrease in the incorporation of ^{14}C-palmitate into $^{14}CO_2$ nd into water-soluble intermediates in the muscle tissue (Table II). In the presence of etate about 60 of the label taken up was recovered in the chloroform extract of the uscle tissue.

On the basis of the oxygen consumption (Table I) and incorporation of ^{14}C-palmitate nto ^{14}CO (Table II), it was calculated that palmitate oxidation accounted for about 7% f the oxidativ metabolism of the perfused muscle (Table II). The addition of acetate did not significantly alter this figure.

Influence of palmitate on U-^{14}C-acetate metabolism

About 40 of the total U-^{14}C-acetate uptak was oxidized to CO_2 and H_2O as judged by he incorporation of label into $^{14}CO_2$, and bout 1% was recovered in the chloroform extract of the muscle tissue (Table III). 46 of the acetate taken up was recovered as water-soluble compounds obtained in the perchloric acid extract of the muscle tissue. Acetate oxidation accounted for 20–30 of the total oxygen consumption. There was no significant change in acetate oxidation or incorporation of ^{14}C-acetate into lipids when palmitate was added (Table

TABLE I Rates of palmitate, acetate and oxygen uptake and net production of lactate in the perfused hindquarter of the rat

Additions to standard medium	Palmitate uptake	Acetate uptake (µmol/h g wet weight)	Oxygen consumption	Net production of lactate
Control[a]	—	—	27.3 ± 1 4 (5)	0 1 + 0 1 (5)
Acetate 2 mM + infusion	—	8.0 ± 0.5 (7)	30 6 ± 1.8 (6)	3[b] 0 1
Palmitate 1 mM	0.94 ± 0 06 (8)	—	24.9 ± 2.0 (10)	2 0[c] ± 0 1
Palmitate, 1 mM Acetate, 2 mM + infusion	1 06 ± 0.07 (10)	7.0 ± 0 4 (8)	26.2 ± 1 1 (10)	3 0[c] + 0 5

Results are expressed as mean ± S.E. with the number of observations in parentheses. Rat hindquarters were perfused for 70 min The standard medium contained glucose (5.5 mM). Substrates were added at min as single doses (palmitate) or as single doses followed by continuous infusion (acetate; 7.4 µmol/h) (Karlsson et al. 1975).
[a] Data taken from Karlsson *et al* (1976).
[b] Significantly different from control $p < 0.05$
[c] As for b, $p < 0.01$

At the end of the perfusion portion of the thigh muscle was rapidly frozen *in situ* with aluminium tongs cooled in liquid nitrogen (Wollenberger *et al* 1960). The frozen tissue (200–350 mg) was homogenized and treated with $HClO_4$ as previously described (Karlsson *et al* 1975). The perchloric acid extract obtained was analyzed for the content of adenosine-triphosphate (ATP) (Lamprecht and Trautschold 1963), adenosine-monophosphate (AMP) (Adam 1962), creatine-phosphate (CP) (Lamprecht and Stein 1963), inorganic phosphate (P_i) (Baginski *et al* 1970) and lactate and pyruvate (Hohorst *et al* 1959)

Radioactivity measurement

The metabolic fates of the added substrates were studied with U-^{14}C-acetate and 1-^{14}C-palmitate. The labelled substrates were added to give a specific activity of 9 nCi/µmol of acetate and 15 nCi/µmol of palmitate in the medium. Added label was recovered at the end of the perfusion as described previously (Karlsson *et al* 1975 Karlsson *et al* 1976) except that radioactivity remaining in the perfusate at the end of the perfusion was measured by counting 1 ml of an aliquot of the plasma sample. Recoveries of radioactivity are given in Tables II and III Uptake of label from the medium was calculated from the change in substrate concentration and the specific activity of the added substrate.

Statistical analysis

Students t-test was used for statistical analysis, values of p 0 05 being considered statistically significant.

Results

Rates of palmitate acetate and oxygen uptake and net production of lactate

The addition of palmitate to the perfusion medium caused slight hemolysis of the red cells but this did not affect the arterial oxygen concentration

Palmitate was added to give a final concentration of 1 mM in the medium and the uptake of palmitate was linear during the experimental period Palmitate uptake was not altered by the addition of acetate (Table I) Acetate was continuously infused into the perfusion medium and the concentration of acetate (2 mM) was essentially constant throughout the experimental period The uptake of acetate from the medium was not significantly altered when palmitate was included (Table I).

Table IV The effect of acetate and palmitate or both on the concentrations of lactate and pyruvate and the [lactate]/[pyruvate] (L/P) ratio in the medium and the muscle tissue of the perfused hindquarter of the rat at the end of the perfusion.

Additions to perfusion medium	Muscle tissue			Medium		
	Lactate (μmol/g wet weight)	Pyruvate	L/P	Lactate (mM)	Pyruvate	L/P
Control[a]	1.15±0.20 (6)	0.14±0.02 (6)	7.6±1.4 (6)	2.18±0.14 (5)	0.11±0.02 (5)	20.7±4.4 (5)
Palmitate, 2 mM[a] infusion	2.21[b]±0.47 (4)	0.08[b]±0.01 (6)	27.4[c]±8.6 (4)	2.04±0.13 (6)	0.13±0.01 (6)	16.2±1.4 (6)
Acetate, 1 mM	3.45[d]±0.12 (9)	0.12±0.02 (9)	29.7[d]±10.1 (9)	1.79[b]±0.14 (10)	0.16[b]±0.01 (10)	11.4[b]±0.8 (10)
Acetate, 1 mM + palmitate, 2 mM infusion	3.00[d]±0.41 (10)	0.11±0.01 (10)	28.3[d]±5.6 (10)	2.27±0.25 (10)	0.18[c]±0.01 (10)	13.2±1.1 (10)

Results are expressed as means ± S.E. with the number of observations in parentheses. Experimental conditions, see Table I and Methods.
[a] Data taken from Karlsson *et al.* (1976)
[b] Significantly different from control perfusion, $p < 0.05$.
[c] As for b, $p < 0.01$
[d] As for b, $p < 0.001$

...trohfeldt *et al.* 1974). In addition, the metabolic fates of 1-^{14}C-palmitate (Table II) (Beatty and Bocek 1971) and 1-^{14}C-oleate (Reimer *et al.* 1975) were essentially the same *i.e.* most ^{14}C-label was found in muscle lipids, while about 10% of the ^{14}C-fatty acid taken up was oxidized to $^{14}CO_2$. These similarities between palmitate and oleate metabolism have also been found in man (Hagenfeldt 1975).

In contrast to palmitate, most ^{14}C-acetate was oxidized to $^{14}CO_2$ and H_2O while only minor amounts of ^{14}C-label were found in lipids (Table III). If the incorporation of label into $^{14}CO_2$ from ^{14}C-palmitate is expressed as μmol of acetate, the figure in Table II will be about 0.6 (0.08 S) μmol of acetate/h g. Thus, there is a 5 times higher incorporation of radioactivity into $^{14}CO_2$ from ^{14}C-acetate, than from ^{14}C-palmitate. Since most palmitate is stored as lipids, while acetate was mainly oxidized, the two compounds enter different metabolic pathways in the muscle cell at rest. A faster oxidation of short-chain fatty acids as compared to long-chain fatty acids, has previously been observed in skeletal muscle (Fritz 1961) and in intact rats *in vivo* (Kirschner and Harris 1961). It is known that the activation of short-chain fatty acids in skeletal muscle is localized within the mitochondrion while the activation of long-chain fatty acids is mainly extra-mitochondrial (Bressler 1970, Aas 1971). Since acetate easily penetrates the mitochondrial membrane (Spencer and Lowenstein 1962), this compound can, after activation to acetyl-CoA, immediately enter the oxidative pathway, i.e. the citric acid cycle. Palmitate, on the other hand, will pass through several regulatory steps (activation, conversion to carnitine derivative, re-activation to acyl-CoA and finally β-oxidation steps) before it enters the citric acid cycle, where $^{14}CO_2$ is formed. In agreement with our results, it has been found previously that palmitate is not used as an immediate energy source in the resting muscle but is mainly incorporated into lipids before

TABLE III The effect of palmitate on the metabolic fate of U-^{14}C-acetate in the perfused hind-q[illegible] the rat.

Additions to standard medium	Acetate uptake (µmol/h g wet weight)	Incorporation of label into $^{14}CO_2$ (µmol of acetate/h g wet weight)	Lipids (µmol of acetate/h g wet weight)	Oxygen consumption due to oxidation of ^{14}C-acetate to $^{14}CO_2$ (%)[b]	Recov[illegible] of [illegible] label (%)
^{14}C-acetate, 2 mM[a] + infusion	8.3±0.4 (6)	3.17±0.22 (6)	0.12±0.04 (6)	30.6±1.7 (6)	91.[illegible]
^{14}C-acetate 2 mM + infusion Palmitate 1 mM	7.9±0.3 (6)	3.65±0.54 (6)	0.13±0.02 (6)	48.5±4.2 (6)	93.[illegible]

Results are expressed as mean±S.E. with the number of observations in parentheses. Experimental [illegible]tions see Table I and Methods.
[a] Data taken from Karlsson *et al* (1976).
[b] Correction has not been made for re-oxidation of NADH by lactate dehydrogenase.

III) but the content of ^{14}C label in the perchloric acid extract was increased to about [illegible] ($p < 0.05$).

Lactate and pyruvate concentrations and L/P ratios in the muscle tissue and the medium at the end of the perfusion

The [lactate]/[pyruvate] (L/P) ratio in the muscle tissue was elevated when palmit[illegible] acetate or both were added to the medium (Table IV). In the presence of palmitate [illegible] changes were mainly due to an increased lactate concentration, since the pyruvate conc[illegible]tion was essentially unchanged (Table IV). However, with acetate as the only added [sub]strate the change in L/P ratio in the muscle tissue was due to an increase in lacta[te and a] decrease in the pyruvate concentration. The changes in L/P ratios in the muscle tissu[e were] not reflected by similar changes in the medium.

Tissue content of adenine-nucleotides, creatine-phosphate and inorganic phosphate and the "energy charge" in the muscle tissue

The tissue content of ATP, ADP, AMP, CP and P_i was not significantly altered [by] addition of acetate as compared to the control (Table V). However, in the presence [of pal]mitate acetate caused a statistically significant decrease in the tissue content of ATP [...] and P_i as compared to the perfusion with palmitate but no acetate (Table V). The ch[anges] in high-energy phosphate compounds and P_i by acetate in the presence of palmitate [were] not reflected by changes in the "phosphate potential" ([ATP]/[ADP] [P_i]) (Atkinson [...]) or the ratio [ADP]^2/[ATP] [AMP] which represents the mass action ratio of the aden[ylate] kinase reaction (Table V).

Discussion

Palmitate uptake in the present study (Table I) was of the same order of magnitude [as the] uptake of oleate in the perfused rat hind-quarter reported by others (Reimer *et al* [...]

IV The effect of acetate and palmitate or both on the concentrations of lactate and pyruvate and the [lactate]/[pyruvate] (L/P) ratio in the medium and the muscle tissue of the perfused hindquarter of the rat at the end of the perfusion.

...ons to ...ard medium	Muscle tissue			Medium		
	Lactate (μmol/g wet weight)	Pyruvate	L/P	Lactate (mM)	Pyruvate	L/P
...rol[a]	1.15 0.20 (6)	0.14 ± 0.02 (6)	7.6 ± 1.4 (6)	2.18 ± 0.14 (5)	0.11 ± 0.02 (5)	20.7 ± 4.4 (5)
...te, 2 mM[a] ...fusion	2.21[b] ± 0.47 (4)	0.08[b] ± 0.01 (6)	27.4[c] ± 8.6 (4)	2.04 ± 0.13 (6)	0.13 ± 0.01 (6)	16.2 ± 1.4 (6)
...tate, 1 mM	3.45[c] ± 0.12 (9)	0.12 ± 0.02 (9)	39.7[c] ± 10.1 (9)	1.79[b] ± 0.14 (10)	0.16[c] ± 0.01 (10)	11.4[c] ± 0.8 (10)
...ate, 1 mM ...te, ...mM ...fusion	3.80[c] ± 0.41 (10)	0.11 ± 0.01 (10)	30.3[c] ± 5.6 (10)	2.27 ± 0.25 (10)	0.18[c] ± 0.01 (10)	13.2 ± 1.1 (10)

...es are expressed as mean ± S.E. with the number of observations in parentheses. Experimental condi-..., see Table I and Methods.
...re taken from Karlsson *et al.* (1976).
...ificantly different from control perfusion, $p < 0.05$.
...for a, $p < 0.01$
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...hfeldt *et al.* 1974). In addition, the metabolic fates of 1-¹⁴C-palmitate (Table II) (Beatty ... Bocek 1971) and 1-¹⁴C-oleate (Reimer *et al.* 1975) were essentially the same *i.e.* most ...label was found in muscle lipids, while about 10% of the ¹⁴C-fatty acid taken up was ...ized to ¹⁴CO_2. These similarities between palmitate and oleate metabolism have also ...n found in man (Hagenfeldt 1975).

...n contrast to palmitate, most ¹⁴C-acetate was oxidized to ¹⁴CO_2 and H_2O while only ...or amounts of ¹⁴C-label were found in lipids (Table III). If the incorporation of label ... ¹⁴CO_2 from ¹⁴C-palmitate is expressed as μmol of acetate, the figure in Table II will ...about 0.6 (0.05 8) μmol of acetate/h g. Thus, there is a 5 times higher incorporation of ...ioactivity into ¹⁴CO from ¹⁴C-acetate, than from ¹⁴C-palmitate. Since most palmitate ...s stored in lipids, while acetate was mainly oxidized, the two compounds enter different ...tabolic pathways in the muscle cell at rest. A faster oxidation of short-chain fatty acids compared to long-chain fatty acids, has previously been observed in skeletal muscle ...tz 1961) and in intact rats *in vivo* (Kirschner and Harris 1961). It is known that the activa-...n of short-chain fatty acids in skeletal muscle is localized within the mitochondrion while ... activation of long-chain fatty acids is mainly extra-mitochondrial (Bremer 1970, Aas ...71). Since acetate easily penetrates the mitochondrial membrane (Spencer and Lowenstein ...62), this compound can, after activation to acetyl-CoA, immediately enter the oxidative ...thways i.e. the citric acid cycle. Palmitate, on the other hand, will pass through several ...[illegible] steps (activation, conversion to carnitine derivative, re-activation to acyl-CoA ...d finally β-oxidation steps) before it enters the citric acid cycle, where ¹⁴CO_2 is formed. ...agreement with our results, it has been found previously that palmitate is not used as an ...mediate energy source in the resting muscle but is mainly incorporated into lipids before

TABLE III The effect of palmitate on the metabolic fate of U-[14]C-acetate in the perfused hind-[illegible] the rat.

Additions to standard medium	Acetate uptake (μmol/h g wet weight)	Incorporation of label into		Oxygen consumption due to oxidation of [14]C acetate to $^{14}CO_2$ (%)[b]	[illegible]
		$^{14}CO_2$ (μmol of acetate/h g wet weight)	Lipids		
[14]C-acetate 2 mM + infusion	8.3±0.4 (6)	3.17±0.22 (6)	0.12±0.04 (6)	20.6±1.7 (6)	[illegible]
[14]C-acetate, mM + infusion Palmitate, 1 mM	7.9±0.3 (6)	3.65±0.54 (6)	0.13±0.02 (6)	28.5±4.2 (6)	[illegible]

Results are expressed as mean ± S.E. with the number of observations in parentheses. Experimental conditions see Table I and Methods.
Data taken from Karlsson et al. (1976)
[b] Correction has not been made for re-oxidation of NADH by lactate dehydrogenase.

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The [lactate]/[pyruvate] (L/P) ratio in the muscle tissue was elevated when palm acetate or both were added to the medium (Table IV). In the presence of palmita changes were mainly due to an increased lactate concentration, since the pyruvate con tion was essentially unchanged (Table IV). However, with acetate as the only add strate the change in L/P ratio in the muscle tissue was due to an increase in lact decrease in the pyruvate concentration. The changes in L/P ratios in the muscle tiss not reflected by similar changes in the medium.

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…IV The effect of acetate and palmitate or both on the concentrations of lactate and pyruvate and the [lactate]/[pyruvate] (L/P) ratio in the medium and the muscle tissue of the perfused hindquarter of the rat at the end of the perfusion.

…ions to …rd medium	Muscle tissue			Medium		
	Lactate (μmol/g wet weight)	Pyruvate	L/P	Lactate (mM)	Pyruvate	L/P
…trol[a]	1.15 ± 0.20 (6)	0.14 ± 0.02 (6)	7.6 ± 1.4 (6)	2.18 ± 0.14 (5)	0.11 ± 0.02 (5)	20.7 ± 4.4 (5)
…tate, 2 mM[a] infusion	2.21[b] ± 0.47 (4)	0.08[b] ± 0.01 (6)	27.4[c] ± 8.6 (4)	2.04 ± 0.13 (6)	0.13 ± 0.01 (6)	16.2 ± 1.4 (6)
…mitate, 1 mM	3.45[c] ± 0.12 (9)	0.12 ± 0.03 (9)	39.7[c] ± 10.1 (9)	1.79[b] ± 0.14 (10)	0.16[b] ± 0.01 (10)	11.4[b] ± 0.8 (10)
…mitate, 1 mM …tate, 2 mM …infusion	3.80[c] ± 0.41 (10)	0.11 ± 0.01 (10)	38.3[c] ± 5.6 (10)	2.27 ± 0.25 (10)	0.18[c] ± 0.01 (10)	13.2 ± 1.1 (10)

…ues are expressed as mean ± S.E. …th the number of observations in parentheses. Experimental condi…ns, see Table I and Methods.
…ata taken from Karlsson *et al.* (1976).
…ignificantly different from control perfusion, p … 0.05.
…s for a, p … 0.01
…s for a, p … 0.001

…rnfeldt *et al.* 1974). In addition, the metabolic fates of 1-…C-palmitate (Table II) (Beatty …d Bocek 1971) and 1-…C-oleate (Renner *et al.* 1975) were essentially the same: … most …C-label was found in muscle lipids, while about 10% of the ¹⁴C-fatty acid taken up was …idized to $^{14}CO_2$. These similarities between palmitate and oleate metabolism have also …een found in man (Hagenfeldt 1975).

In contrast to palmitate, most …C-acetate was oxidized to …CO… and H_2O while only …inor amounts of ¹⁴C-label were found in lipids (Table III). If the incorporation of label …nto $^{14}CO_2$ from ¹⁴C-palmitate is expressed as μmol of acetate, the figure in T…ble II will …e about 0.6 (0.08 · 8) μmol of acetate/h · g. Thus, there is a 5 times higher incorporation of …adioactivity into $^{14}CO_2$ from …C-acetate, than from ¹⁴C-palmitate. Since most palmitate …as stored as lipids, while acetate was mainly oxidized, the two compounds enter different …metabolic pathways in the muscle cell at rest. A faster oxidation of short-chain fatty acids …as compared to long-chain fatty acids, has previously been observed in skeletal muscle (Fritz 1961) and in intact rats *in vivo* (Kirschner and Harris 1961). It is known that the activation of short-chain fatty acids in skeletal muscle is localized within the mitochondrion while the activation of long-chain fatty acids is mainly extra-mitochondrial (Bressler 1970, Aas 1971). Since acetate easily penetrates the mitochondrial membrane (Spencer and Lowenstein 1962), this compound can, after activation to acetyl-CoA, immediately enter the oxidative pathways … the citric acid cycle. Palmitate, on the other hand, will pass through several regulatory steps (activation, conversion to carnitine derivative, re-activation to acyl-CoA and finally β-oxidation steps) before it enters the citric acid cycle, where ¹⁴CO… is formed. I… agreement with our results, it has been found previously that palmitate is not used as an immediate energy source in the resting muscle, but is mainly incorporated into lipids before

TABLE III The effect of palmitate on the metabolic fate of U-^{14}C-acetate in the perfused hind-quarter of the rat.

Additions to standard medium	Acetate uptake (μmol/h g wet weight)	Incorporation of label into $^{14}CO_2$ (μmol of acetate/h g wet weight)	Incorporation of label into Lipids (μmol of acetate/h g wet weight)	Oxygen consumption due to oxidation of ^{14}C-acetate to $^{14}CO_2$ (%)[b]	Recovery of added label (%)
^{14}C-acetate, 2 mM[a] + infusion	8.3±0.4 (6)	3.17±0.22 (6)	0.12±0.04 (6)	20.6±1.7 (6)	91±4
^{14}C-acetate, 2 mM + infusion Palmitate 1 mM	7.9±0.3 (6)	3.65±0.54 (6)	0.13±0.02 (6)	28.5±4.2 (6)	98±4

Results are expressed as mean±S.E. with the number of observations in parentheses. Experimental conditions see Table I and Methods.

[a] Data taken from Karlsson et al (1976).

[b] Correction has not been made for re-oxidation of NADH by lactate dehydrogenase.

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The [lactate]/[pyruvate] (L/P) ratio in the muscle tissue was elevated when palm acetate or both were added to the medium (Table IV). In the presence of palmit changes were mainly due to an increased lactate concentration, since the pyruvate co tion was essentially unchanged (Table IV). However with acetate as the only ad strate the change in L/P ratio in the muscle tissue was due to an increase in lac decrease in the pyruvate concentration. The changes in L/P ratios in the muscle tis not reflected by similar changes in the medium.

Tissue content of adenine-nucleotides creatine-phosphate and inorganic phosphate and the "energy charge" in the muscle tissue

The tissue content of ATP ADP AMP CP and P_i was not significantly altere addition of acetate as compared to the control (Table V). However in the presenc mitate acetate caused a statistically significant decrease in the tissue content of AT and P_i, as compared to the perfusion with palmitate but no acetate (Table V). The in high-energy phosphate compounds and P_i by acetate in the presence of palmit not reflected by changes in the "phosphate potential" ([ATP]/[ADP] [P_i]) (Atkinso or the ratio [ADP]^2/[ATP] [AMP] which represents the mass action ratio of the ad kinase reaction (Table V).

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Palmitate uptake in the present study (Table I) was of the same order of magnitud uptake of oleate, in the perfused rat hind-quarter reported by others (Reimer et a

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...itions to ...dard medium	Muscle tissue			Medium		
	Lactate (μmol/g wet weight)	Pyruvate	L/P	Lactate (mM)	Pyruvate	L/P
...l[a]	1.13 ± 0.20 (6)	0.14 ± 0.02 (6)	7.6 ± 1.4 (6)	2.18 ± 0.14 (5)	0.11 ± 0.03 (5)	20.7 ± 4.4 (5)
...e, 2 mM[a] ...ment	2.21[b] ± 0.47 (4)	0.08[b] ± 0.01 (4)	27.4[c] ± 8.6 (4)	2.04 ± 0.13 (6)	0.13 ± 0.01 (6)	16.2 ± 1.4 (6)
...tate, 1 mM	3.45[d] ± 0.12 (9)	0.12 ± 0.02 (9)	39.7[d] ± 10.1 (9)	1.79[b] ± 0.14 (10)	0.16[b] ± 0.01 (10)	11.4[b] ± 0.8 (10)
...tate, 1 mM ...e, mM ...ation	3.80[d] ± 0.41 (10)	0.11 ± 0.01 (10)	38.3[d] ± 5.6 (10)	2.27 ± 0.23 (10)	0.18[c] ± 0.01 (10)	13.2 ± 1.1 (10)

...s are expressed as mean ± S.E. with the number of observations in parentheses. Experimental conditions see Table I and Methods.
...a taken from Karlsson et al. (1976).
...ificantly different from control perfusion, $p < 0.05$.
...or a, $p < 0.01$
...ter a, $p < 0.001$

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...contrast to palmitate most ^{14}C-acetate was oxidized to $^{14}CO_2$ and H_2O while only ...t amounts of ^{14}C-label were found in lipids (Table III). If the incorporation of label ...$^{14}CO_2$ from ^{14}C-palmitate is expressed as μmol of acetate, the figure in Table II will ...bout 0.6 (0.08 8) μmol of acetate/h g. Thus, there is a 5 times higher incorporation of ...activity into $^{14}CO_2$ from ^{14}C-acetate, than from ^{14}C-palmitate. Since most palmitate ...stored as lipids, while acetate was mainly oxidized, the two compounds enter different ...abolic pathways in the muscle cell at rest. A faster oxidation of short-chain fatty acids ...compared to long-chain fatty acids, has previously been observed in skeletal muscle ...tz 1961) and in intact rats in vivo (Kirschner and Harris 1961). It is known that the activa... of short-chain fatty acids in skeletal muscle is localized within the mitochondrion while ...activation of long-chain fatty acids is mainly extra-mitochondrial (Bremer 1970, Aas ...1). Since acetate easily penetrates the mitochondrial membrane (Spencer and Lowenstein ...2), this compound can, after activation to acetyl-CoA, immediately enter the oxidative ...hways i.e. the citric acid cycle. Palmitate, on the other hand, will pass through several ...ulatory steps (activation, conversion to carnitine derivative, re-activation to acyl-CoA ...l finally β-oxidation steps) before it enters the citric acid cycle, where $^{14}CO_2$ is formed. ...agreement with our results, it has been found previously that palmitate is not used as an ...mediate energy source in the resting muscle, but is mainly incorporated into lipids before

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TABLE V The effect of acetate and palmitate or both on the concentrations of adenine-nucleotides, creatine phosphate and inorganic phosphate and the energy-charge of the resting, perfused hind-quarter at the end of the perfusion.

Additions to standard medium	ATP (μ mol/g wet weight)	ADP	AMP	CP	Pi	$\frac{ATP}{ADP \cdot Pi}$	$\frac{ADP}{ATP \cdot AMP}$
Control[a]	5 01 ± 0.31 (6)	0.70 ± 0 05 (6)	0.024 ± 0.006 (6)	16.4 ± 1.5 (6)	16.0 ± 1.2 (6)	0 46 ± 0.03 (4)	5 5 + 2 5 (4)
Acetate 2 mM[a] + infusion	5 33 ± 0.16 (6)	0.77 ± 0 09 (6)	0.026 ± 0.004 (5)	16.0 ± 1 6 (5)	15 1 ± 1 6 (6)	0.51 ± 0 07 (6)	5 3 ± 1 4 (5)
Palmitate, 1 mM	5.93[b] ± 0 16 (8)	0.77 ± 0 04 (8)	0 024 ± 0.004 (6)	17.3 ± 1.5 (8)	14.1 ± 1.3 (8)	0 59 ± 0 09 (8)	5 4 + 1 9 (4)
Palmitate, 1 mM Acetate, 2 mM + infusion	5 03[f] ± 0..6 (10)	0.67[e] ± 0.04 (10)	0.019 ± 0.003 (5)	15.2 ± 1.2 (10)	10 5[c] ± 0.6 (9)	0.69 ± 0 03 (9)	5 5 ± 1 2 (5)

Results are expressed as mean ± S.E. with the number of observations in parentheses. Experimental conditions, see Table I and Methods.

[a] Data taken from Karlsson *et al.* (1976).
[b] Significantly different from control perfusion, $p < 0.05$
[c] As for *a* $p < 0.01$
[d] As for *a*, $p < 0.001$
[e] Significantly different from perfusion with palmitat but without acetate, $p < 0.05$.
[f] As for *d* $p < 0.01$

it is oxidized (Issekutz 1970 Havel 1970) This might explain the finding that, in spite of excessive exogenous substrates, the oxidation of palmitate and acetate together accounts for only 35% of the total oxygen consumption in the perfused tissue (Table II and I). Reimer *et al* (1975) found that most label from 1 [14]C-oleate incorporated into lipids in perfused rat hind-quarter was found in the triglyceride fraction.

The addition of acetate to the medium caused a 40% decrease in the incorporation of label from [14]C palmitate into $^{14}CO_2$ and into water-soluble intermediates in the muscle tissue (Table II). The concomitant tendency of an increase in the amount of label in the lipid extract of the muscle tissue in the presence of acetate indicates that the [14]C-palmitate was diverged to lipid synthesis. Although this effect was not statistically significant, it could explain the decreased incorporation of C-palmitate into $^{14}CO_2$, since the inhibition would only affect the distribution of palmitate between the oxidative and synthetic reactions by about 3%, if the incorporation of label into $^{14}CO_2$ is expressed as per cent of palmitate uptake (Table II) Furthermore we did not find any evidence of competition between palmitate and acetate at the same enzyme site since changes in -C acetate metabolism were seen when palmitate was added and the simultaneous presence of the two substrates did not affect the uptake of either of them Thus, at present we are not able to stipulate the exact site of interaction of acetate on 1 [14]C-palmitate oxidation, but one possible explanation may be a competition between the two substrates for CoA There are no published studies on the interaction between short-chain and long-chain fatty acids in skeletal muscle at rest, and previous studies on rat heart have given conflicting results. Shipp (1964) found that in the perfused rat heart acetate did not affect palmitate uptake but inhibited palmitate oxidation, thus confirming our findings in skeletal muscle On the other hand Bethencourt

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The increase in lactate production (Table IV), caused by the addition of acetate or palmi-te or both, was followed by an increase in the tissue concentration of lactate and a corre-ponding elevation of the L/P ratio in the tissue (Table V). These changes were not reflected y similar changes in the medium. Reimer *et al.* (*op. cit.*) also found, with the same experi-mental technique, that the addition of oleate caused no changes in the L/P ratio in the per-sion medium.

In conclusion, acetate affected palmitate metabolism only slightly if the effect is related o the total palmitate uptake by the muscle. However there was 40% inhibition of palmi-tte oxidation, which indicates a profound effect of acetate on palmitate oxidation to CO_2. At present the site of this inhibition is not known. Extrapolation of our findings to the situa-ion in vivo indicates that acetate will not contribute to an altered lipid metabolism in other ssues through its action on skeletal muscle. However the present results will probably not eflect the situation in the stimulated skeletal muscle, since in this situation the energy equirements will increase and the importance of long-chain fatty acids for the oxidative metabolism is enhanced (Keul *et al.* 1972). Thus, any inhibition of the oxidative metabolism of long-chain fatty acids may be of great significance during exercise.

Thanks are due to Miss G.-B. Jönsson for skilful technical assistance. This work was financially supported by Svenska Carl Gerschanskys minnesfond, the Faculty of Mathematics and Science of the University of Uppsala and by Grant no. B74-25X-00575-12 from the Swedish Medical Research Council.

References

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TABLE V The effect of acetate and palmitate or both on the concentrations of adenine-nucleotides, creatine phosphate and inorganic phosphate and the "energy-charge" of the resting, perfused hind-quarter at the end of the perfusion.

Additions to standard medium	ATP (μmol/g wet weight)	ADP	AMP	CP	Pi	ATP / ADP Pi	ADP / ATP AMP
Control[a]	5 01±0.31 (6)	0.70±0.05 (6)	0.024±0.006 (6)	16.4±1.5 (6)	16.0±1.2 (6)	0 46±0 03 (6)	55 [illegible] (6)
Acetate, 2 mM + infusion	5.33±0.16 (6)	0.77±0 09 (6)	0.026±0.004 (5)	16 0±1 6 (5)	15 1±1 6 (6)	0 51±0 07 (6)	53±1[illegible] (5)
Palmitate, 1 mM	5 93[b]±0.16 (8)	0.77±0 04 (8)	0.024±0.004 (6)	17 3±1.5 (8)	14 1±1.3 (8)	0.59±0 09 (8)	56 19 (6)
Palmitate, 1 mM Acetate 2 mM + infusion	5.03[f]±0.[illegible]6 (10)	0 67[e]±0 04 (10)	0 019±0.003 (5)	15.2±1.2 (10)	10.5[c,e]±0.6 (9)	0.69±0 03 (9)	55+1[illegible] (5)

Results are expressed as mean±S.E. with the number of observations in parentheses. Experimental conditions see Table I and Methods.

[a] Data taken from Karlsson *et al* (1976).

[b] Significantly different from control perfusion, $p < 0.05$

[c] As for [b] $p < 0.01$

[d] As for [a] $p < 0.001$

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[f] As for [d], $p < 0.01$

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J. Interrelation between carbohydrate and fatty acid metabolism of isolated perfused rat heart. *Arzneimittel-Forschung* 1964, *13*, 852–864.

A. F. and J. M. Lowenstein, The supply of precursors for the synthesis of fatty acids. *J. biol. Chem.* 1962, *237* 3640–3648.

P. H. Kettl and K. F. Weinges, Perfusion of the isolated rat hindlimb with synthetic medium. *Horm. Metab. Res.* 1974, *6*, 167–168.

H. and H. Barry III, *Actions of alcohol*, vol. 1–2. Elsevier Publ. Comp. Amsterdam, London, New York 1970.

A., O. Rietau and G. Schmitt, Eine einfache Technik der extrem schnellen Abkühlung größerer Gewebestücke. *Pflügers Arch. ges. Physiol.* 1960, *270*, 399–412.

ADAM, H Adenosin-5-diphosphat und Adenosin-5-monophosphat. In *Methoden der Enzym. Analyse* Ed. by H U Bergmeyer Verlag Chemie, Weinheim/Bergstr 1962.
ATKINSON D E., In *Metabolic roles of citrate* Ed. by T W Goodwin, Academic Press, London
BAGINSKI, E. S. P P FOA and B ZAK, Glucose-6-phosphatase. In *Methoden der Enzymatischen A* Ed. by H -U Bergmeyer Verlag Chemie, Weinheim/Bergstr 1970.
BEATTY C. H and R. M BOCEK, Interrelation of carbohydrate and palmitate metabolism in skeletal muscle. *Amer J Physiol.* 1971 220 1928–1934
BETHENCOURT A. V O E. MATOS and J C. SHIPP Acetate-1 ^{14}C metabolism and the effect of glucose and palmitate metabolism in the perfused rat heart. *Metabolism* 1966. 15 847–851
BERGMEYER, H U and H MÖLLERING, Acetat. Bestimmung mit vorgeschalteter Indikatorreaktion. *Methoden der Enzymatischen Analyse* Ed. by H. U Bergmeyer Verlag Chemie. Weinheim/Bergstr 1970.
BRESSLER, R Fatty acid oxidation. In *Comprehensive biochemistry* Vol. 18. Lipid metabolism. Ed. M Florkin and E. H Stoltz. Elsevier Publ. Comp., Amsterdam. 1970.
CEDERBAUM, A. I. and E. RUBIN, Differential effects of acetate on palmitate and octanoate oxidation: segregation of acetyl CoA pools. *Arch Biochem.* 1975. 166 618–628.
DUNN, R. B. and J B CRITZ, Uptake of lactate by dog skeletal muscle *in vivo* and the effect of free fatty acids. *Amer J Physiol.* 1975 229 255–259
FRITZ, I B Factors influencing the rates of long-chain fatty acid oxidation and synthesis in mammalian systems. *Physiol. Rev* 1961 41 52 129
HAGENFELDT L., Turnover of individual free fatty acids in man. *Fed. Proc* 1975. 34 2246–2249.
HAVEL, R. J Lipid as energy source. In. *The physiology and biochemistry of muscle as a food*, 2. Ed. E. J Briskey R. C. Cassens and B. B. Marsh. The University of Wisconsin Press. Madison, Milwaukee and London. 1970.
HOHORST H J F H KREUTZ und TH BÜCHER, Über metabolitgehalte und Metabolit-Konzentrationen in der Leber der Ratte. *Biochem. Z.* 1959 332 18–46.
ISSEKUTZ, B., JR., Interrelationship of free fatty acids, lactic acid and glucose in muscle metabolism. *The physiology and biochemistry of muscle as a food*, 2. Ed by E. J Briskey R. G Cassens and Marsh. The University of Wisconsin Press. Madison, Milwaukee and London. 1970.
ISRAEL, Y and J MARDONES (eds.), *Biological basis of alcoholism.* Wiley-Interscience. New York, London, Sidney Toronto. 1971
ITAYA, K and M UI, Colorimetric determination of free fatty acids in biological fluids. *J Lipid Res.* 6 16–20.
KARLSSON, N E. FELLENIUS and K. H. KIESSLING, The metabolism of acetate in the perfused hind-quarter of the rat. *Acta physiol scand* 1975 93 391–400
KARLSSON N E. FELLENIUS and K. H KIESSLING, Influence of acetate on glucose metabolism in the perfused hind-quarter of the rat. *Acta physiol scand.* 1976. 98. 347 355
KEUL, J E. DOLL and D KEPPLER, In *Energy metabolism in human muscle* Medicine and sport, Ed. by E. Jokl. University Park Press. Baltimore, London, Tokyo. 1972.
KIRSCHNER, S. L. and R S. HARRIS, The effects of chain length on the metabolism of saturated fatty acids by the rat. *J Nutr* 1961 73 397–402.
KISSIN, B and H. BEGLEITER (eds.), *The biology of alcoholism.* Vol. 1 3 Plenum Press. New York, London 1971
KREBS, H A. and K. HENSELEIT Untersuchungen über die Harnstoffbildung im Tierkörper *Hoppe-Seylers Z. physiol. Chem.* 1932. 210 33–66.
LAMPRECHT W and P STEIN Creatinphosphat I *Methoden der Enzymatischen Analyse* Ed. by Bergmeyer Verlag Chemie, Weinheim/Bergstr 1962.
LAMPRECHT W und J TRAUTSCHOLD Adenosine-5-triphosphat. Bestimmung mit Hexokinase und Zwischenferment. In *Methoden der Enzymatischen Analyse* Ed. by H. U Bergmeyer Verlag Chemie, Weinheim/Bergstr 1962.
LEE, J S. and G V VAHOUNY Compartmentalized oxidative pathways in rat heart mitochondria. *Proc.* 1974 33 685
LIEBER, C. S., Effects of ethanol upon lipid metabolism. *Lipids* 1974 9 103–116.
LUNDQUIST F., Production and utilization of free acetate in man. *Nature* (Lond.) 1962. 193 579–58
REIMER, E. S G LÖFFLER, G. HENNIG and O. H. WIELAND, The influence of insulin glucose and acid metabolism in the perfused rat hindquarter *Hoppe-Seylers Z. physiol.* 1975 356. 1055–

J. Interrelation between carbohydrate and fatty acid metabolism of isolated perfused rat heart. Metabolism 1964, *13* 852–866.

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Acta physiol. scand. 1977 99 166–172
From the Department of Physiology University of Göteborg, Sweden

Contractile Responses to Noradrenaline. Varying Dependence on External Calcium of Consecutive Vascular Segments of Perfused Rat Hindquarters

By

MORLEY C. SUTTER, MARGARETA HALLBÄCK, JOHN V JONES and BJÖRN FOLKOW

Received 29 June 1976

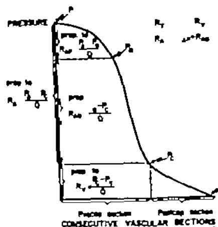
Abstract

SUTTER, M C., M HALLBÄCK, J V JONES and B FOLKOW *Contractile responses to noradrenaline Varying dependence on external calcium of consecutive vascular segments of perfused rat hindquarters.* Acta physiol. scand. 1977 99 166–172.

To study the dependency of vascular smooth muscle contractions, produced by noradrenaline, on the concentration of extracellular calcium, experiments were performed on adult male Wistar rats. The hemodynamic characteristics of the isolated, perfused hindquarters were investigated from maximal dilatation up to maximal, noradrenaline-induced constriction under constant flow conditions. Responses to noradrenaline in the consecutive segments of the vascular bed ("proximal" and distal precapillary resistance vessels and postcapillary resistance vessels) were determined at low (0 mM) and at normal (1.5–2.0 mM) Ca^{++} concentrations in the perfusate Dependence on external calcium is greater peripherally The smallest pre and postcapillary resistance vessels are the most dependent while the larger "proximal" precapillary resistance vessels are the least dependent on the availability of external calcium. The results illustrate the considerable differentiation of smooth muscle, depending on its location along the vascular circuit. They further indicate that it is likely that the hemodynamically so important microvessels are highly dependent on extrinsic Ca^{++} sources not only concerning their involvement in remote adrenergic control, but also in their maintenance of normally pronounced "myogenic" tone.

It is known that *in vitro* various types of smooth muscle in larger vessels differ considerably in several important respects, including their dependence on external calcium (Ca^{++}) during contraction to noradrenaline (NA). At one extreme is the aorta which continues to contract to NA in the absence of external Ca^{++} and at the other is the portal-mesenteric vein which will not contract without external Ca^{++} This raises the question as to which is the most appropriate analogue of the pre- and postcapillary microvessels responsible for the important resistance and capacitance adjustments which in the end determine the hemodynamic characteristics.

On sabbatical leave, Dept. of Pharm. Univ of British Columbia, Vancouver, Canada.
From Dept. of Med. Cardiology Univ of Glasgow Scotland.



1 Schematic representation of the resistance to … along … vascular circuit illustrating the pressure … across the consecutive resistance sections. The … at … which pressures were measured or calculated … the perfused rat hindquarter are indicated by P_a, P and P_v, respectively (see text)

There is, however, … paucity of information about requirements for extracellular Ca^{++} … vascular smooth muscle contractions produced by NA *in situ*. Haddy (1960) studied the … fects of increased Ca^{++} on responses to NA of the blood vessels of the perfused dog fore… limb. Jones *et al.* (1973) and Hinke (1966) have reported the effects of reduced Ca^{++} on re… sponses to NA in rat mesenteric vascular preparations and tail artery, respectively. How… ever, both of these preparations may be more representative in several respects of … conduit … arteries … than of the true resistance vessels lying adjacent to the capillaries. As it seems … important to gain more information about the functional characteristics of these important … microvessels, experiments were performed to examine the effects of very low external Ca^{++} … concentrations on the respective dose response curves to NA in the consecutive vascular … segments of perfused rat hind quarters. The results indicate that the dependence on external … Ca^{++} increases considerably as one proceeds peripherally in the vascular tree with the smallest … pre- and postcapillary vessels being most and larger arteries least dependent.

A brief report based on some of these results has been presented elsewhere (Folkow *et al.* 1976)

Methods

The results are compiled from perfusion of the hindquarters of 26 male Wistar rats … with an artificial plasma substitute as described previously (Folkow *et al.* 1970, 1974) but utilizing Dextran, 4%, instead of Ficoll as colloid. In each … preparation the rise in arterial inflow pressure (P …) in response to … g NA infusion reflects the changes in total resistance to flow (R_T) of the hindquarter vascular bed and … as measured in all animals. T… differentiate between the responses of the larger, more proximal and smaller, more distal precapillary vessels, pressure (P …) was also measured in … small limb artery (external diameter at maximum dilatation 200–300 μm) in 6 animals. Under constant flow conditions P … P … then gives … measure of … "proximal" precapillary resistance (R_{Ap}) and its relationships to the total resistance to flow (R_T).

In 9 of the 26 rats an aplethysmographic technique (Folkow *et al.* 1974) was used to differentiate between responses of pre- and postcapillary resistance vessels, by measuring arterial inflow pressure (P_A), venous outflow pressure (P …) and tissue volume. Mean capillary pressure (P_C), and hence pre- and postcapillary resistances (R … and R_V, respectively) could then be calculated by quantitative evaluations of the changes in capillary filtration produced by mechanically and transiently altering inflow and outflow pressures,

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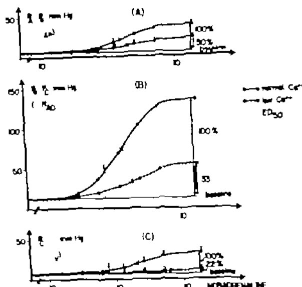
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3. Legends as for Fig. 2, except: A) P is pressure measured in a small artery with an external radius ... relaxation (... been exposed to locally applied papaverine) around 0.1–0.15 (see text). $P_A - P$ is proportional to R_{AP} ("proximal" precapillary resistance) since flow was kept constant throughout ... and vertical bars represent M ± S.E. from 6 animals. B) P – P is proportional to R_{AD} ("distal" ...apillary resistance). The points are values calculated from the curves shown in Fig. 2, 3A and 3C. ... P ... proportional to postcapillary venous resistance. The points represent M ± S.E. from 8 or 9 ...als. As in Fig. 2 the vertical dashed line in A, B and C indicates ... noradrenaline concentration of ... 10^{-7} M ... the horizontal dashed line demarcated by ... arrows indicates how much the noradrenaline ...centration must be increased at low calcium to elicit the same pressor response.

... infusion coincide closely with observations in the intact rat as to the relative magnitude ... the resistance increases in the "proximal" resistance vessels. They imply that the normal ...tive control of resistance is not only localized to the distal microvessels but involves a ...latively extensive portion of the precapillary vessels, including their larger "proximal" ...sels as well. There is also ... clear tendency for functional differentiation among the proxi...al and distal vessels in the rat. The proximal resistance vessels evidently contribute relatively ...ore to the centrally directed vasoconstrictor fibre control than to the locally originated "myogenic" resistance, which consequently resides mainly in the distal precapillary resistance ...sels under basal conditions in vivo though, of course, with a considerable spatial overlap.

Concerning the situation during in vitro perfusion the concentration of Ca^{++} in the venous effluent was from 0.4 to 0.5 mM during perfusion with low Ca^{++} solutions (0.2 mM). The pressor responses to increasing concentrations of NA in consecutive segments of the vascular bed, perfused with normal or low Ca^{++} solutions, are shown in Fig. 2 and 3. Low external Ca^{++} reduced the responsiveness of each portion of the vascular bed and shifted the ED_{50} for NA to the right (toward higher concentrations of NA) in each section.

However quantitatively the effects of low Ca^{++} on the NA concentration–effect curves differed considerably in the consecutive segments of the vasculature, which could be directly

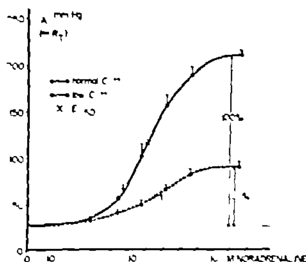


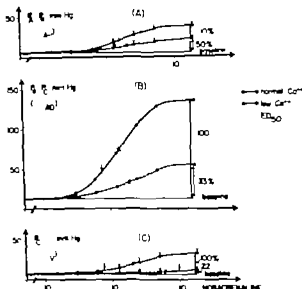
Fig. — Concentration-effect curve of noradrenaline on arterial inflow pressure in isolated, perfused rat hindquarters, normal or low calcium in the perfusate. The vertical dashed line indicates a noradrenaline concentration of 7.5 10^{-7} M; the lower dashed line demarcated by arrows indicates how much the noradrenaline concentration must be increased at low calcium to elicit the same pressor response. The individual points and vertical bars represent M ± S.E. from 4 to 13 animals.

respectively (*cf.* Folkow *et al.* 1974, Eliasson *et al.* 1974). Knowing R_A and R_{AP} the resistance of "distal" precapillary resistance vessels (R_{AD}) could be deduced as well. The resistance drop along the vascular tree is schematically shown in Fig. 1 which also indicates in general principle the segments in which measurements were made or calculated.

Pressor responses to NA, at concentrations in the perfusion fluid from 1.5 10^{-7} to 10^{-4} M, were measured at low (0. mM) and at normal (1.5 mM) Ca^{++} concentration in the perfusate. Concentration-effect curves for NA were then constituted for pressor responses of the total vascular bed and the consecutive segments of the vascular bed, *i.e.* the "proximal" and "distal" precapillary resistance vessels and the postcapillary resistance vessels. Since flow was kept constant the changes in pressure drop along these consecutive segments reflect directly the respective changes in their resistances to flow.

Results

The resistance to flow offered by the "proximal" resistance vessels (R_{AP}) makes up about 25 % of R_T in the perfused hindquarter vascular bed during maximal vasodilatation and about 20 during maximal constriction, as long as Ca is normal (Fig. compare Fig. 3 A). To show that this relatively high contribution of R_{AP} to R_T during constriction is not merely due to the circumstances of perfusion, with a diffuse distribution of high concentrations and a consequent artificially intense involvement of proximal "small arteries" the changes in $P_A - P$ also were followed when the rats were still intact with normal blood perfusion and vasoconstrictor fibre control. During basal *in vivo* conditions $P_A - P$ accounted for some 10 of the total systemic pressure drop, implying that "distal" precapillary resistance vessels (R_{AD}) then contributed 70-75 %, if it is assumed that mean capillary pressure is around 15 mmHg in this situation (*cf.* Eliasson *et al.* 1974) and central venous pressure close to zero. However the contribution to total pressure drop ($P_A - P_V$) of $P_A - P$ increased from about 10 to some 25 upon intense vasoconstrictor fibre discharge, which was elicited as a reflex response to graded withdrawal of blood. Since flow in the steady state situation is always the same in the consecutive segments of a vascular circuit, it follows from this observation that the "proximal" resistance vessels of the rat are relatively more strongly constricted by the vasoconstrictor fibres than the "distal" ones. Thus the observations during hindquarter perfusion with plasma substitute and noradre



3 Legends as for Fig 2, except A) P is pressure measured in small artery with an external radius complete relaxation (when exposed to locally applied papaverine) around 0.1–0 15 (see text) $P_A - P$ is A proportional to R_{AP} ("proximal" precapillary resistance) since flow was kept constant throughout. nts and vertical bars represent M ± S E. from 6 animals B) P − P is proportional to R_{AD} ("distal" capillary resistance) The points are values calculated from the curves shown in Fig. 2, 3 A and 3 C. P P_V is proportional to postcapillary venous resistance The points represent M ± S E. from 8 or 9 animals. As in Fig 2 the vertical dashed line in A, B and C indicates noradrenaline concentration of 10^{-7} M the horizontal dashed line demarcated by arrows indicates how much the noradrenaline concentration must be increased at low calcium to elicit the same pressor response.

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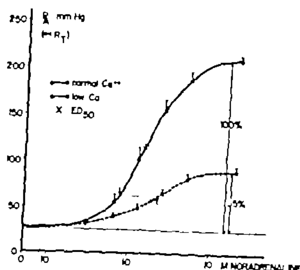


Fig. 2. Concentration-effect curve of adrenaline on arterial inflow pressure in isolated perfused rat hindquarters with normal or low calcium in the perfusate. The vertical dashed line indicates a noradrenaline concentration of 7.5 10⁻ M; the lower dashed line demarcated by arrows relates how much the noradrenaline concentration must be increased at low calcium to obtain the same pressor response. The individual points and vertical bars represent M ± S.E. from 4 to 13 animals.

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	(1) P_A		(2) P – P		(3) P – P		(4) P – P	
	At ED_{50}	At 2.5 10^{-4} M	At ED_{50}	At 2.5 10^{-4} M	At ED_{50}	At 2.5 10^{-4} M	At ED_{50}	At 2.5 10^{-4} M
ormal	100	100	100	100	100	100	100	100
ow	34	29	50	48	33	26	19	9

nse (P) responses to noradrenaline at ED_{50} and at 2.5 10^{-4} M. (Response in normal Ca^{++} = 100.) he ED_{50} responses allow comparisons to be made at the same point on the concentration–effect curve, not necessarily at the same concentration of noradrenaline, since low concentrations of calcium t the ED_{50} toward higher concentrations of noradrenaline. The concentration of 2.5 10^{-4} M was osed since this on the linear part of the concentration–effect curve. Regardless of which type of parison made the larger, proximal resistance vessels ($P_A - P_a$) retain their responses to noradrenaline ter in low calcium than the distal precapillary resistance vessels (P – P_O) or the postcapillary resistance els (P P)

Discussion

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These in our findings that dependence on external calcium for vascular smooth muscle contractility to NA increases as one proceeds from central to peripheral vessels are consistent with similar suggestions based on comparisons of the canine aorta, carotid and mesenteric arteries in an *in vitro* study of the effects of neomycin on Ca^{++} binding and distribution (Goodman *et al.* 1975). It should further be stressed that in most vascular circuits, the smallest precapillary resistance vessels normally display a particularly pronounced myogenic tone, and these "spontaneous" contractions must reasonably also be highly dependent on Ca influx. I fact, they are presumably even more dependent on extrinsic Ca^{++} than when NA induces contractions because NA appears t facilitate the mobilization of Ca^{++}. It is therefore likely that this hemodynamically most important inherent tone of the smallest precapillary resistance vessels is particularly much depressed by reductions in Ca^{++} concentration.

It is of primary interest to know which of the several types of larger vessels commonly used *in vitro* that constitutes the most appropriate model of microvascular sections responsible for resistance, capillary flow distribution and capacitance, including dependence on external Ca^{++}. Two commonly used vessels for *in vitro* studies are rat thoracic aorta and portal vein. I such characteristics as inherent activity, propagation of excitation and con

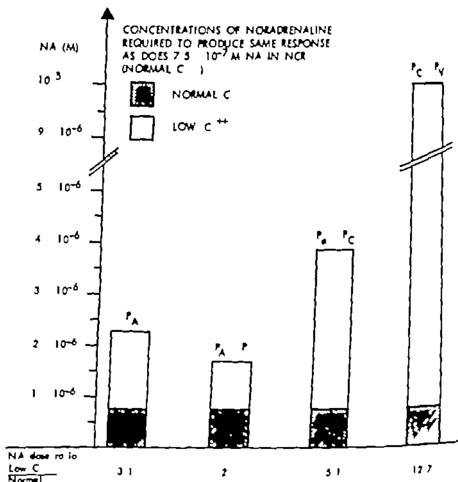


Fig. 4 Comparison of the concentrations of noradrenaline which are equipressor in hindquarters perfused with normal or low calcium solutions. P_A represents the pressor (resistance) changes in the hindquarter vascular circuit as a whole, $P_A - P$ and $P_a - P_0$ those in the proximal and distal precapillary resistance vessels, respectively and $P_0 - P_V$ those in the postcapillary resistance vessels.

estimated from the pressure drops since flow was kept constant (Fig. 4 and Table I). Fig. shows the NA concentrations which are *equipressor* in low and normal Ca perfusions and the ratios of one to the other. A comparison of these ratios shows that a reduced external calcium concentration leads to a far more extensive depression of the NA responses in the immediately pre- and postcapillary segments ($P - P_0$ and $P_0 - P_V$) than in the larger vessels ($P_A - P$). Thus, the NA concentration need only be doubled to produce the same pressor response in the proximal precapillary vessels, whereas it must be increased some 5- to 1 -fold to produce equipressor responses in the distal small pre- and postcapillary vessels, respectively.

Table I compares pressor responses to NA with low or normal Ca in the perfusate at two points in the concentration effect curves at the ED_{50} and at an arbitrary NA concentration of 2.5 10^{-4} M. Again, low Ca^{++} is seen to reduce the responses to NA considerably more in the small pre- and postcapillary segments than in the larger upsteam vessels (Columns (2) or (3) or (4))

1

	(1) P		(2) $P_A - P$		(3) $P - P$		(4) $P_O - P_V$	
	At ED_{50}	At 2.5×10^{-6} M	At ED_{50}	At 2.5×10^{-6} M	At ED_{50}	At 2.5×10^{-6} M	At ED_{50}	At 2.5×10^{-6} M
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These in situ findings that dependence on external calcium for vascular smooth muscle contractility to NA increases as one proceeds from central to peripheral vessels are consistent with similar suggestions based on comparisons of the canine aorta, carotid and mesenteric arteries in an in vitro study of the effects of neomycin on Ca^{++} binding and distribution (Goodman et al. 1975). It should further be stressed that in most vascular circuits, the smallest precapillary resistance vessels normally display a particularly pronounced myogenic tone, and these "spontaneous" contractions most reasonably also be highly dependent on Ca^{++} influx. In fact, they are presumably even more dependent on extrinsic Ca^{++} than when NA induces contractions because NA appears to facilitate the mobilization of Ca^{++}. It is therefore likely that this hemodynamically most important inherent tone of the smallest precapillary resistance vessels is particularly much depressed by reductions in Ca^{++} concentration.

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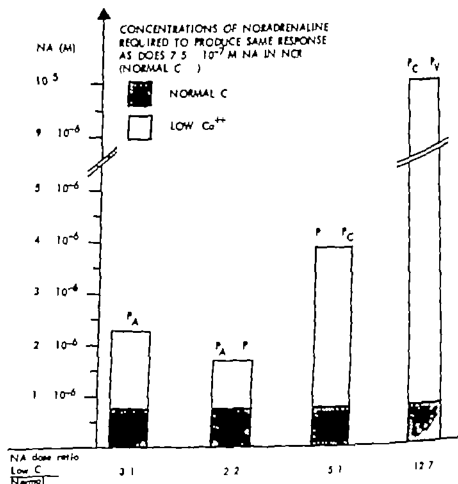


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	(1) P		(2) P → P		(3) P → P		(4) $P_O - P_V$	
	At ED_{50}	At 2.5 × 10^{-4} M	At ED_{50}	At 2.5 × 10^{-4} M	At ED_{50}	At 2.5 × 10^{-4} M	At ED_{50}	At 2.5 × 10^{-4} M
[illegible]rmal	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
[illegible]w	34	29	50	43	33	26	19	9

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strictor fibre influence the smooth muscle of the microvessels appears to be more sim to that of the portal vein than to that of the aorta (e.g. Ljung 1970, Rhodes and Sutter 19 Concerning the dependence on external Ca It has been found (Sutter 1976) that the presence of 0.4 mM external Ca^{++} aortic strips retain 90% and portal vein only 40% of their maximal response to NA in normal Ca^{++}. It seems reasonable to sugg that 0.4 mM Ca^{++} is similar to the concentration of Ca^{++} achieved in our hindquarter p fusions since the inflow and outflow concentrations were 0.2 and 0.4–0.5 mM, respective The portal vein in its behaviour in low Ca^{++} then is remarkably similar to the precapillary resistance vessels (here corresponding to $P-P_O$) which retain 33% of t maximal responses to NA and also similar to the postcapillary resistance vessels (P_C) (Table I column 3). It seems that the mesenteric-portal vein is in several important resp a better analogue of the true resistance vessels than is aortic smooth muscle, as has b suggested previously (Ljung 1970 Rhodes and Sutter 1971) and according to the pre findings this is true also with regard to sources of Ca Furthermore at any given lev low Ca^{++} the myogenic contractions of the portal vein are far more depressed than t induced by NA (Sutter 1976). This is in line with the suggestion mentioned above precapillary myogenic tone, which normally seems to be the dominant element in the m tenance of the systemic resistance to flow is likely to be particularly depressed by ductions of Ca^{++} in the immediate chemical environment.

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References

ELIASSON E., B. FOLKOW, S. M. HILTON, B. ÖBERG and B. RIPPE. Pressure-volume characteristics interstitial fluid space in the skeletal muscle of the cat. *Acta physiol. scand.* 1974. 90. 583–593.

FOLKOW B., M. HALLBÄCK, J. JONES and M. SUTTER, Effects of noradrenaline on consecutive segments at low or normal calcium concentrations in NCR and SHR. *Clin. Sci. Mol. Med.* 1976. In

FOLKOW B. M. HALLBÄCK, Y. LUNDGREN and L. WEISS, Background of increased flow resistance vascular reactivity in spontaneously hypertensive rats. *Acta physiol. scand.* 1970. 80. 93–106.

FOLKOW B. M. HALLBÄCK, Y. LUNDGREN, L. WEISS, I. ALBRECHT and S. JULIUS, Analysis of design reactivity of series-coupled vascular sections spontaneously hypertensive rat (SHR). *Acta ph scand.* 1974. 90. 654–656.

GOODMAN, F. R., H. R. ADAMS and G. B. WEISS, Effects of neomycin on ^{45}Ca binding and distributi canine arteries. *Blood Vessels* 1975. 12. 240–260.

HADDY F. J. Local effects of sodium, calcium and magnesium upon small and large blood vessels dog forelimb. *Circulat. Res.* 1960. 8. 57–70.

HINKE, J. A. Effect of Ca upon contractilities of small arteries from DCA-hypotensive rats. *Cir Res.* 1966. 18. Suppl. 1. 23–34.

JONES, D. R. B. M. LYON and E. L. PHELAN, Calcium ions and vascular reactivity in the genet hypertensive rat. *Proc. of Univ. Otago med. Sch.* 1973. 51. 30–31.

LJUNG, B., Nervous and myogenic mechanisms in the control of vascular neuroeffector system physiol. scand. 1970. Suppl. 349. 33–68.

RHODES, H. J. and M. C. SUTTER, The action of diazoxide on isolated vascular smooth muscle ele physiology and contraction. *Can. J. Physiol. Pharmacol.* 1971. 49. 276–287.

SUTTER, M. C., Quantitative effects of external calcium concentration on contraction of rat portal compared to thoracic aorta. *Acta physiol. scand.* 1976. 98. 266–268.

Acta physiol. scand. 1977 99 173–182

From the Department of Physiology University of Goteborg, Sweden

Time Course and Extent of Carotid Sinus Baroreceptor Threshold Resetting in Rats with Renovascular Hypertension

By

JOHN V JONES

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Abstract

J V *Time course and extent of carotid sinus baroreceptor threshold resetting in rats ith renovascular hypertension.* Acta physiol. scand. 1977 99 173–182.

iy the extent and time course of arterial baroreceptor threshold resetting to increases in blood pres-nal hypertension was induced in young normotensive male Wistar rats by unilateral renal artery ping. At different intervals after operation the extent of baroreceptor threshold resetting in the l sinus as estimated. Experiments were performed 7 14 and 25 days after renal artery clipping to baroreceptor resetting to be correlated with the rate and extent of cardiovascular changes in rats had renovascular hypertension of identical durations (Lundgren *et al.* 1974). Baroreceptor thresh-n the carotid sinuses were established by progressive clamping of both carotid arteries after partial ir isolation of the sinuses. The results show that after one week of hypertension baroreceptor resetting y just apparent but by two weeks resetting is gross and seems to be largely completed. This parallels laptive changes in the hindquarters of renal hypertensive rats and it is concluded that baroreceptor ng secondary phenomenon related to the structural changes induced in the vessels by the elevated pressure

e is general agreement that the arterial baroreceptors are reset to the higher blood pres-levels in hypertensive animals and man. This was clearly demonstrated in dogs with renal ertension by McCubbin, Green and Page (1956) and has been abundantly confirmed e then (Aars 1968, Angell-James 1973) in a variety of species including spontaneously ertensive rats (Nosaka and Wang 1972, Sapru and Wang 1976). Various different mech-ms have been postulated to account for baroreceptor resetting and include altered di-ensibility of the receptor area (Aars 1969), degeneration of the receptor endings (Kezdi 7 Angell-James 1973) or combination of the two. Central mechanisms may of course, important in the overall picture of resetting but do not explain alterations in impulse ffic in the carotid sinus and aortic nerves themselves.

Resetting of aortic baroreceptors may occur extremely rapidly in acutely induced hyper ension (Krieger 1970). It is also present in carotid sinus baroreceptors a few days after the set of renal hypertension in dogs (McCubbin 1958). However the exact time course of this

Present address University Department of Medical Cardiology Royal Infirmary Glasgow Scotland.

strictor fibre influence the smooth muscle of the microvessels appears to be more re to that of the portal vein than to that of the aorta (*e.g* Ljung 1970, Rhodes and Sutter 1 Concerning the dependence on external Ca^{++} it has been found (Sutter 1976) the presence of 0 4 mM external Ca^{++} aortic strips retain 90% and portal vein only 40% of their maximal response to NA in normal Ca^{++} It seems reasonable to that 0 4 mM Ca^{++} is similar to the concentration of Ca^{++} achieved in our hindquarte fusions since the inflow and outflow concentrations were 0.2 and 0.4–0.5 mM, respec The portal vein in its behaviour in low Ca^{++} then is remarkably similar to the precapillary resistance vessels (here corresponding to $P - P_0$) which retain 33% of maximal responses to NA and also similar to the postcapillary resistance vessels (T (Table I column 3) It seems that the mesenteric-portal vein is in several important r a better analogue of the true resistance vessels than is aortic smooth muscle, as h suggested previously (Ljung 1970 Rhodes and Sutter 1971), and according to the p findings this is true also with regard to sources of Ca^{++} Furthermore at any given low Ca^{++} the myogenic contractions of the portal vein are far more depressed tha induced by NA (Sutter 1976) This is in line with the suggestion mentioned abo precapillary myogenic tone, which normally seems to be the dominant element in th tenance of the systemic resistance to flow is likely to be particularly depressed ductions of Ca^{++} in the immediate chemical environment.

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References

ELIASSEN, E., B FOLKOW S. M HILTON, B ÖBERG nd B. RIPPE, Pressure-volume characteristi interstitial fluid space in the skeletal muscle of the cat *Acta physiol scand* 1974 90 583–59

FOLKOW B M HALLBÄCK, J JONES nd M SUTTER, Effect of noradrenaline on consecutive segments at low or normal calcium concentrations in NCR and SHR *Clin. Sci Mol Med* 1976

FOLKOW B., M HALLBÄCK, Y LUNDGREN a d L. WEISS, Background of increased flow resista vascular reactivity in spontaneously hypertensive rats. *Acta ph siol scand* 1970. 80 93–106.

FOLKOW B., M HALLBÄCK, Y LUNDGREN, L. WEISS, I ALBRECHT and S. JULIUS, Analysis of des reactivity of series-coupled vascular sections in spontaneously hypertensive rats (SHR) *Acta scand* 1974. 90 654–656.

GOODMAN, F R H R. ADAMS and G B. WEISS, Effects of neomycin on ^{45}Ca binding and distrib canine arteries. *Blood Vessels* 1975 I 240–260.

HADDY F J Local effects of sodium, calcium and magnesium upon small and large blood vessel dog forelimb. *Circulat Res* 1960. 8 57–70.

HINKE, J A., Effect of Ca upon contractilities of small arteries from DCA hypotensive rats. *Res.* 1966. 18 ppl. I 23–34

JONES, D R B M LYON and E L PHELAN, Calci m ions nd vascular reactivity in the ge hypertensive rat. *Proc of Univ Otago med Sch* 1973 51 30–32.

LJUNG, B., Nervous and myogenic mechanisms in the co trol of vascular neuroeffector system *physiol scand* 1970. Suppl. 349 33–68

RHODES, H J and M C. SUTTER, The ction of diazoxide on isolated vascula smooth muscle physiology and contraction. *Can. J Ph siol. Pharmacol* 1971 49 276–287

SUTTER, M C., Quantitative effect of external calcium concentration on contraction of r t port and thoracic aorta. *Acta ph siol. scand.* 1976. 98 266–68.

Acta physiol. scand. 1977 99 173–182

From the Department of Physiology University of Göteborg, Sweden

Time Course and Extent of Carotid Sinus Baroreceptor Threshold Resetting in Rats with Renovascular Hypertension

By

JOHN V JONES[1]

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Abstract

J V *Time course and extent of carotid sinus baroreceptor threshold resetting in rats [wi]th renovascular hypertension*. Acta physiol. scand. 1977 99 173–182.

[Fo]r the extent and time course of arterial baroreceptor threshold resetting to increases in blood pres[sure ...]ed hypertension was induced in young normotensive male Wistar rats by unilateral renal artery [clipp]ing. At different intervals after operation the extent of baroreceptor threshold resetting in the [carotid sinus] was estimated. Experiments were performed 7 14 and 23 days after renal artery clipping to [allow b]aroreceptor resetting to be correlated with the rate and extent of cardiovascular changes in rats [with] renovascular hypertension of identical durations (Lundgren *et al.* 1974). Baroreceptor thresh[olds of] the carotid sinuses were established by progressive clamping of both carotid arteries after partial [o]r isolation of the sinuses. The results show that after one week of hypertension baroreceptor resetting [is] just apparent but by two weeks resetting is gross and seems to be largely completed. This parallels [the ada]ptive changes in the hindquarters of renal hypertensive rats and it is concluded that baroreceptor [resett]ing [is a] secondary phenomenon related to the structural changes induced in the vessels by the elevated pressure.

[It] is general agreement that the arterial baroreceptors are reset to the higher blood pres[sure l]evels in hypertensive animals and man. This was clearly demonstrated in dogs with renal [hyper]tension by McCubbin, Green and Page (1956) and has been abundantly confirmed [since] then (Aars 1968, Angell–James 1973) in a variety of species including spontaneously [hype]rtensive rats (Nosaka and Wang 1972, Sapru and Wang 1976). Various different mech[anis]ms have been postulated to account for baroreceptor resetting and include altered di[stens]ibility of the receptor area (Aars 1969), degeneration of the receptor endings (Kezdi [196?,] Angell–James 1973) or a combination of the two. Central mechanisms may of course, [be i]mportant in the overall picture of resetting but do not explain alterations in impulse [traf]fic in the carotid sinus and aortic nerves themselves.

[R]esetting of aortic baroreceptors may occur extremely rapidly in acutely induced hyper[ten]sion (Krieger 1970). It is also present in carotid sinus baroreceptors a few days after the [o]nset of renal hypertension in dogs (McCubbin 1958). However the exact time course of this

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resetting in relation to the development of chronic hypertension is unknown. It is clear to what extent resetting is secondary to structural vascular changes conseque the hypertension.

In this study the time course and extent of carotid sinus baroreceptor threshold has been studied in rats with experimental renovascular hypertension In such rats and extent of blood pressure rise and the secondary cardiovascular structural following renal artery clipping are known (Lundgren *et al* 1974) It should the possible to correlate in such animals the extent of structural vascular adaptation extent of baroreceptor resetting.

Methods

In order to produce renovascular hypertension in 6–7 week old male Wistar rats, a silver clip (0.2 nal diameter) was placed on the left renal artery under brief ether anesthesia. The right kidn intact. In this way about 50 per cent of the operated animals developed hypertension of sufficie degree as to be included in this study Animals were not considered to be hypertensive unless mean blood pressure in excess of 145 mmHg.

On the day of the baroreceptor threshold study the degree of hypertension was determined by intraarterial blood pressure measurement in the caudal rtery The tail artery was cannulated d ether anaesthesia after which the arterial blood pres ure and heart rate were meas red when was fully awake The heart rate and blood pressure in a control group of animals were determ identical way The caudal artery was also used for measurement of the systemic arterial pressure experiment Pressures were recorded by means of Statham pressure transducers and all data wer on a Grass model 7 polygraph

A control group of animals was studied as were animals with renovascular hypertension of 25 days duration. 6 animals were included in each group

After determination of awake heart rate and blood pressure the animals were then anaesthe more with ether The right femoral vein was cannulated and chloralose (60 mg/kg) as admini venously whereupon the ether was discontinued The animal was placed on its back and all four l down. A midline incision was made from mandible to sternum. The skin and subcutaneous tis tracted and a tracheal cannula was inserted The trachea and oesophagus were di ided cranial to tion of the tracheal cannula nd retracted This allowed easier access to the carotid bifurcation both sides dependi g on the direction of retraction of the trachea nd oesophagus. Both superior nerves were di ided s were both hypoglossal nerves and hyoid bones. This latter manoeuver greater access to the external carotid arteries which were then both cannulated and connected t pressure transducers. All branches of the external carotid arteries were ligated but great care avoid damage to the carotid bifurcation regio s themselves. The internal carotid nd occipi were left intact nd undisturbed The common carotid arteries were exposed on both sides from the carotid inus regions as possible The vagi and cervical sympathetic trunks were left int clamps were placed round the common carotid rteries on both ides. By tighteni g the clamps th distal t the clamps could be reduced nd the new pressure levels recorded in the cannulae in th carotid arteries. The extent t which the carotid si pres re could be lowered depended upon of retrograde f ill g of the sinus. In the event I no nimal was retrograde filling so great as the determination of a threshold for carotid sinus baroreceptor activation, *i e* the pressure in could always be lowered f enough despite the internal carotid artery being left intact.

The nimals were paralysed with gallamine (10 mg/kg I) and put on intermittent positi ventilation by means of a Starling pump. The nimal were ventilated with ga mi t re conta CO_2 in O_2. Anesthesia was maintained where necessary w th intravenous chloralose and additic mine (10 mg/kg I) was given every 20 min. The nimals were allowed to st bilise for 90 min afte tion of the surgery before mesurement of the baroreceptor threshold was made

Carotid sinus pressure w s reduced I teps of pproximately 20 mmHg by djustment of bo artery clamps. As clamping of one carotid rtery commonly aused rise of blood pres re and I... in the opposite sinus pressure some manipulation of the clamps wa ecessary bef

Mean values ± S E for renal hypertensive rats (RHR) 7 14 and 25 days after renal artery constric tion and group of normotensive controls (NCR) concerning body weight, awake arterial blood pressure, left ventricular weight and percentage left ventricular weight/body weight

	Arterial blood pressure, mmHg	Body weight, g	Left ventricular weight, g	Percentage left ventricular weight/body weight
	99.0 ± 3.9	235 ± 14.6	0.50 ± 0.033	0.213 ± 0.008
days	157.3 ± 1.9	214 ± 8.0	0.55 ± 0.033	0.258 ± 0.011
4 days	165.6 ± 3.5	230 ± 7.3	0.64 ± 0.021	0.282 ± 0.007
5 days	166.0 ± 6.0	271 ± 8.6	0.74 ± 0.026	0.287 ± 0.003

stable mean pressures could be obtained In addition the rise in blood pressure consequent upon the al sinus pressure reduction often caused secondary rise in sinus pressure with some oscillation of blood pressure as result. This was particularly noticeable at the first step where blood pressure re ses tended to be greatest The sinus pressures and blood pressure were allowed to stabilise at new for as long as it took to obtain reasonably settled values. This was usually at least one minute and often longer Sinus pressures at each step were adjusted to be as near each other as possible although ten proved difficult to have the pressures absolutely the same Pressure differences between the two es were therefore accepted provided they were not greater than 10 mmHg. The step-wise reduction nus pressure was repeated several times in each animal to allow confirmation of the threshold pressure e made

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Results

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The response of the arterial blood pressure to changing the mean carotid sinus pressure shown for all 4 rats in Fig 1–4 When carotid sinus pressure is lowered arterial blood ressure rises. Subsequently as the sinus pressure is reduced to threshold values and lower here are no further increases in blood pressure. However because of the wide individual ariation in blood and sinus pressures, for statistical evaluation of the results, the blood ressure attained in each animal at each step was expressed as percentage of the maximum blood pressure attained in each animal, *i.e.* as a percentage of the blood pressure at the threshold value for baroreceptor activation. Table II shows the results obtained in this way in each group using 2 separate pressure response curves in each animal Mean carotid sinus pressure has been grouped in 15 mmHg blood pressure intervals and the percentage blood pressures compared, using the t-test, with the blood pressure group in the lowest sinus pressure range of each group. I this way the threshold pressure for carotid sinus baroreceptor activation in the normotensive group of rats was found t be in the range 70–84 mmHg. In

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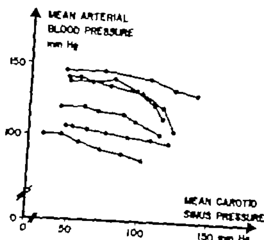


Fig. 1

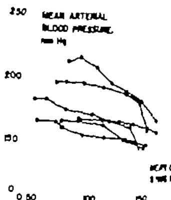


Fig. 2

Fig. 1 The effect on the mean arterial blood pressure of lowering the mean carotid sinus pr normotensive control rats (NCR).

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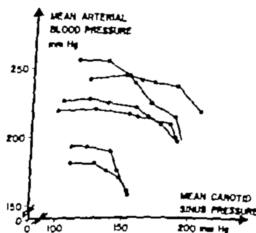


Fig. 3

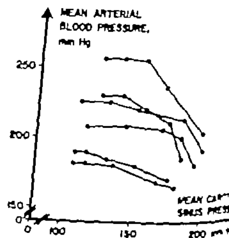


Fig. 4

Fig. 3 The effect on the mea rterial blood pressure of lowering the mea carotid sinus pressure rats (RHR) 14 days after renal artery constriction.

Fig. 4 The effect on the mean arterial blood pressure of lowering the mean carotid sinus pressure rats (RHR) 25 days after renal artery constriction.

Mean values ± S.E. for renal hypertensive rats (RHR) 7, 14 and 25 days after renal artery constriction and group of normotensive controls (NCR) concerning percentage maximum blood pressure attained in each animal. CSP = carotid sinus pressure.

mmHg	NCR	Percentage maximum blood pressure RHR 7 days	RHR 14 days	RHR 25 days
[illegible]	99.8 ± 0.1	—	—	—
[illegible]	98.7 ± 0.4 ns	99.9 ± 0.1	—	—
[illegible]	96.6 ± 0.9 p 0.001	99.2 ± 0.5 ns	—	—
[illegible]	93.1 ± 1.0 p 0.001	97.4 ± 0.9 p 0.001	—	—
[illegible]	90.2 ± 1.2 p 0.001	95.2 ± 1.2 p 0.001	99.0 ± 0.3	99.7 ± 0.3
[illegible]	86.8 ± 2.1 p 0.001	91.3 ± 1.5 p 0.001	99.4 ± 0.3 ns	100 ± 0.0 ns
[illegible]	—	89.6 ± 1.1 $p<0.001$	98.6 ± 0.4 ns	99.0 ± 0.6 ns
[illegible]	—	83.0 ± 2.3 $p<0.001$	94.9 ± 1.2 $p<0.01$	96.7 ± 0.8 $p<0.001$
[illegible]	—	—	92.5 ± 2.1 p 0.001	93.8 ± 1.4 $p<0.001$
[illegible]	—	—	91.7 ± 1.7 p 0.001	90.0 ± 2.0 p 0.001
[illegible]		—	84.9 ± 2.1 p 0.001	85.8 ± 2.6 p 0.001

d to be reset to higher levels when the blood pressure elevation was greater this is e reason for the threshold differences observed between the 7-day hypertensive group e 14 and 25-day hypertensive groups. Fig. 5 shows pressure responses from animals milar resting awake blood pressures but with hypertension of different duration. In imal with hypertension of 7 days duration the threshold lies between 93 mmHg and nHg while the threshold in the animal with hypertension of 14 days duration lies be 137 mmHg and 125 mmHg.

Discussion

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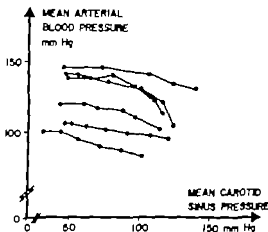


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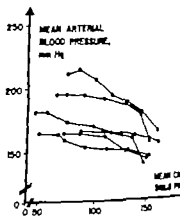


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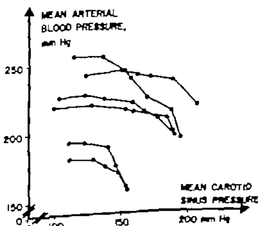


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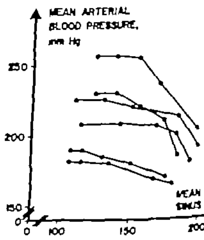


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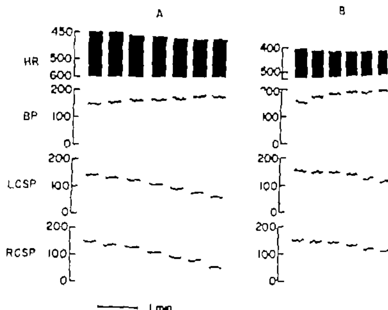


Fig. 5 The effect of loweri g mean pressure in both carotid sinuses on the blood pressure (BP) rate (HR) in A—a rat 7 days after renal artery clipping and B—a rat 14 days after renal art LCSP = left carotid sinus pressure, RCSP = right carotid sinus pressure.

form of adaptation of the receptor has been postulated Indeed there is good evid the baroreceptor reflex may be centrally altered during sleep (Smyth, Sleight and 1969) exercise (Sleight *et al* 1971) and during alertness when defence reactions ar (Hilton 1963 Kylstra and Lisander 1970) The majority view of resetting in hyp however favours some form of structural change in either the nerve endings or the regions or both Aars (1969) has shown less distensibility of the aortic arch in hyp rabbits. This altered distensibility results, it is postulated, in splinting of the bar endings and could account by itself for the resetting in hypertension. However ot described the presence of degenerating nerve endings in both man (Abraham 1967 berg 1967) and in experimental animals (Angell James 1973) Rees (1968) who has the structure of the carotid sinus in some detail with the electron microscope wa to find any evidence of degenerating nerve endings in the carotid sinuses of dogs wit renal hypertension (Sleight *et al* 1975)

The time course of resetting of the baroreceptors is largely unknown Krieger (1 shown that in rats where the aorta was clamped resetting occurs early in this acute hypertension and the majority of aortic arch baroreceptors have reset within 48 h. also shown that reversal of the baroreceptor resetting in rats with renovascular h sion, where the hypertension is abolished by removal of the renal artery clip, is ev rapid. In this latter study the aortic arch baroreceptors had shown reversal of resetti in a matter of hours. This is, of course at variance with the majority view where r has been attributed to physical changes in either the vessel wall or the baroreceptor themselves. Resetting does appear to occur quite rapidly however as it can als detected within a few days of the onset of renal hypertension in dogs (McCubbi and rabbits (Aars 1968). The results of the present study indicate that the resettin

l sinus baroreceptors has the same time course as the development of structural ts, hich are themselves secondary to the rise in blood pressure consequent upon ing one renal artery. It implies that the baroreceptor resetting may be largely secondary se changes although the contribution of central mechanisms cannot be excluded.

ndgren *et al.* (1974) investigated the exact time course and extent of cardiovascular ural adaptation following the rise in mean arterial blood pressure after induction of ascular hypertension in normotensive rats. At different time intervals after induction novascular hypertension the haemodynamic characteristics of the renal hypertensive ed of normotensive control rats were explored using a paired hindquarter perfusion ique. The extent of left ventricular hypertrophy was also examined. It was shown that entricular hypertrophy was present after one week while adaptive structural changes in istance vessels were only just appearing. The cardiovascular structural adaptation ared to be largely completed 2–3 weeks after the operation since no further significant ase in blood pressure, left ventricular hypertrophy or medial hypertrophy occurred this time. It should be emphasised that under these experimental conditions the blood sure elevation was comparatively gradual taking 7–14 days to reach a stable maximum l.

he time course of the changes in the hindquarter vascular bed appear to parallel the re ing of the baroreceptors in rats with renal hypertension. Resetting was slight, but present, one week when the blood pressure is already grossly elevated and approaching a stable ue. At this time structural changes, are only just beginning to appear in the resistance sels although left ventricular hypertrophy is more clearly apparent. The baroreceptors pear to be completely reset after two weeks as there is no difference in threshold values at and 25 days. This also parallels Lundgren *et al.* (1974) findings of the time course of cardio-scular adaptation following renal artery clipping.

It has not been known previously whether the baroreceptors reset passively with the rise blood pressure in chronic hypertension or whether they precede or follow the rise in blood essure. Krieger (1970) has shown that following aortic clamping in the rat the aortic baro-ceptors first show signs of resetting at about 12 h and most of the f bres examined had reset 48 h. This does not, of course, exclude a structural basis for the resetting as such gross anges in load might well induce changes in aortic structure within this time or in aortic all water content with resultant altered distensibility. It is known that skeletal muscle will ypertrophy within this time course in the face of extreme increases in work load (Goldberg 967). What is more difficult to explain is the observation, again by Krieger (Salgado and rieger 1973), that the reversal of renal hypertension causes an almost immediate, albeit ncomplete, reversal of baroreceptor resetting. This observation would suggest that structural hanges may not be so significant at least for the reversal of baroreceptor resetting although f the nerve endings has degenerated it is doubtful whether they could regenerate so rapidly.

McCubbin (1958) has indicated that in chronic hypertension of considerable duration the baroreceptors are tending still to oppose the hypertension. However within the limits of this study the results hav shown the baroreceptor threshold to be completely reset after 2 weeks of renovascular hypertension. At this stage the threshold appears to be nearer the awake mean blood pressure of the animals than was the case in the control animals or in those with

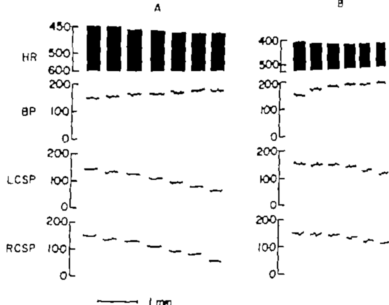


Fig. 5 The effect of lowering mean pressure in both carotid sinuses on the blood pressure (BP) and heart rate (HR) in A—a rat 7 days after renal artery clipping and B—a rat 14 days after renal artery clipping. LCSP = left carotid sinus pressure RCSP = right carotid sinus pressure.

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References

. H. Aortic baroreceptor activity in normal and hypertensive rabbits. *Acta physiol. scand.* 1968. 72, –309

. H. Relationship between aortic diameter and aortic baroreceptor activity in normal and hyperten- e rabbits. *Acta physiol. scand.* 1969. 75. 406–414.

...MAN, A. The structure of baroreceptors in pathological conditions in man. *Baroreceptors and Hyper- sion.* Ed. P. Kezdi. Pergamon Press, New York. 1967. pp. 273–291.

...ALL JAMES, J. E., Characteristics of single aortic and right subclavian baroreceptor fiber activity in bbits ith chronic renal hypertension. *Circulat. Res.* 1973. 32. 149–161

...ER, T. J., G. W. BRADLEY and M. J. PURVES, The relation between carotid body chemoreceptor dis- arge, carotid sinus pressure and carotid body venous flow. *J. Physiol.* (Lond.). 1970. 208. 99–120.

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Gribbin *et al* (1971) have shown that baroreceptor sensitivity declines with incr age However it is unlikely that there would be any significant difference in barorec sensitivity between the rats in this study as they were all young animals. Further the control rats were of ages spanning the age and weight ranges of the hypertensive r and no difference in threshold values was noted between the older normotensive co and the younger control rats.

Occlusion of both common carotid arteries in the rat is often fatal and is associated cerebral ischemia in this species (Thant *et al* 1969 Fujishima *et al.* 1975). However technique employed in this study with gradual reduction in carotid sinus pressure and oc sion of the common carotid artery did not seem to produce untoward effects. Furthermo a plateau in the blood pressure response was obtained before total occlusion and when clamps were closed completely no further effects were noted This would seem also exclude any chemoreceptor influence on the response as this would also have been prog sive with reduction in the sinus pressure Furthermore the rats were ventilated with al pure oxygen, thus minimising chemoreceptor influences while Biscoe, Bradley and Pur (1970) have shown that chemoreceptor afferent traffic is independent of sinus pressure o the range 60–160 mmHg in cats In only the normotensive and 7-day hypertensive an was it necessary to go below this pressure level to obtain a definite threshold for barorece activity There have however been no studies on the relationship between carotid si pressure and chemoreceptor discharge in hypertensive animals. Progressive clamping of t carotid arteries could, in theory have resulted in increased peripheral resistance which co be different in hypertension when compared to normotension This should, of course ha been progressive until complete occlusion of both common carotid arteries was attai which was not the response obtained

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References

rs, H. Aortic baroreceptor activity in normal and hypertensive rabbits. *Acta physiol. scand.* 1968. 72. 58–409

as, H. Relationship between aortic diameter and aortic baroreceptor activity in normal and hyperten- ive rabbits. *Acta physiol. scand.* 1969. 75. 406–414

ABAN, A. The structure of baroreceptors in pathological conditions in man. *Baroreceptors and Hyper- tension* Ed. P. Kezdi. Pergamon Press. New York. 1967. pp. 273–291

ULL-JAMES, J. E. Characteristics of single aortic and right subclavian baroreceptor fiber activity in rabbits with chronic renal hypertension. *Circulat. Res.* 1973. 32. 149–161

COE, T. J., G. W. BRADLEY and M. J. PURVES. The relation between carotid body chemoreceptor dis- charge, carotid sinus pressure and carotid body venous flow. *J. Physiol.* (Lond.) 1970. 208. 99–120.

arrow, D. J., A. J. HONOUR, G. W. PICKERING, P. SLEIGHT and H. S. SMYTH. Diminished baroreflex sensitivity in high blood pressure. *Circulation* 1969. 39. 48–54.

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References

...ARS, H. Aortic baroreceptor activity in normal and hypertensive rabbits. *Acta physiol. scand.* 1968, 72, ...: 298–309.

...ARS, H. Relationship between aortic diameter and aortic baroreceptor activity in normal and hypertensive rabbits. *Acta physiol. scand.* 1969, 75, 406–414.

ABRAHAM, A. The structure of baroreceptors in pathological conditions in man. *Baroreceptors and Hypertension.* Ed. P. Kezdi. Pergamon Press. New York. 1967. pp. 273–291.

ANGELL JAMES, J. E. Characteristics of single aortic and right subclavian baroreceptor fiber activity in rabbits ... chronic renal hypertension. *Circulat. Res.* 1973, 32, 149–161.

BISCOE, T. J., G. W. BRADLEY and M. J. PURVES. The relation between carotid body chemoreceptor discharge, carotid sinus pressure and carotid body venous flow. *J. Physiol.* (Lond.) 1970, 208, 99–120.

BRISTOW, D. J., A. J. HONOUR, G. W. PICKERING, P. SLEIGHT and H. S. SMYTH. Diminished baroreflex sensitivity in high blood pressure. *Circulation* 1969, 39, 48–54.

FOLKOW B M HALLBÄCK Y LUNDGREN, R. SIVERTSSON and L. WEISS, Importance of adaptive ... in vascular design for establishment of primary hypertension, studied in man and in spontaneously hypertensive rats. *Circulat Res* 1973 32 33 Suppl. I I 2 I 13

FUJISHIMA, M T SUGI, Y MOROTOMI and T OMAE, Effects of bilateral carotid artery ... lactate and pyruvate concentrations in normotensive and spontaneously hypertensive rats. ... 6 62–66.

GOLDBERG, A L. Work induced growth of skeletal muscle in normal and hypophysectomized ... *J Physiol* 1967 213 1193–1198

GRIBBIN, B. T G PICKERING, P SLEIGHT and R PETO, Effect of age and high blood pressure ... reflex sensitivity in man. *Circulat Res* 1971 29 424–431

HILGENBERG, F Neurohistologic studies of the carotid sinus baroreceptors in hypertension. ... *and Hypertension* Ed P Kezdi Pergamon Press. New York 1967 pp 293–296.

HILTON S M Inhibition of baroreceptor reflexes on hypothalamic stimulation. *J Physiol* (L... 165 56–57

KEZDI, P Resetting of the carotid sinus in experimental renal hypertension. *Baroreceptors ... tension*. Ed. P Kezdi Pergamon Press. New York 1967 pp. 301 306.

KIRCHHEIM, H R Systemic arterial baroreceptor reflexes. *Physiol Rev* 1976. 56 100–176.

KORNER, P I M J WEST J SHAW and J B. UTHER, "Steady-state properties of the barore... rate reflex in essential hypertension in man *Clin exp Pharmacol Physiol* 1974 1 65–76.

KRIEGER, E. M Neurogenic hypertension in the rat. *Circulat Res* 1964 15 511 521

KRIEGER, E. M Time-course of baroreceptor resetting in acute hypertension. *Amer J Physiol* 486–490.

KYLSTRA, P and B. LISANDER, Differentiated interaction between the hypothalamic defence are... recepto reflexes. *Acta physiol scand* 1970. 78 386–392.

LUNDGREN Y M HALLBÄCK L WEISS and B FOLKOW Rate and extent of adaptive ca... changes in rats during experimental enal hypertension *Acta physiol scand* 1974. 91 103–1...

McCUBBIN J W Carotid sinus participation in experimental renal hypertension. *Circulat...* 791–797

McCUBBIN, J W J H GREEN and I H PAGE, Barorecepto function in chronic renal hypert... *culat Res*. 1956. 4 205– 10

NOSAKO, S. and K. OKAMOTO, Modified characteristics of the aortic baroreceptor activities i... taneously hypertensive rat. *J p Circulat J* 1970. 34 685–693

NOSAKO, S and S. C. WANG, Carotid si us baroreceptor functions in the spontaneously hyp... *Amer J Physiol*. 1972. 222. 1079–1084

REES, P M Electron microscopical observations o the architecture of the carotid arterial ... special reference to the sinus portion. *J Anat* (Lond.). 1968 103 35–47

SALGADO, H C. and E. M KRIEGER, Reversibil ty of baroreceptor daptation in chronic h... *Clin. Sci. Mol. Med.* 1973 45 123–126.

SAP U H N and S. C. WANG, Modification of aortic baroreceptor resetting in the spontane... tensive rat. *Amer J Physiol* 1976. 230 664–674

SLEIGHT P B. GRIBBIN and T G PICKERING Baroreflex sensitivity in normal nd hyperten... effect of beta-adrenergic blockade on reflex sensitivity *Postgrad. med. J* 1971 47 79–83

SLEIGHT P J L. ROBINSON D E. BROOKS and P M REES, Carotid baroreceptor resetting ... tensive dog. *Clin. Sci Mol. Med.* 1975 48 261 263

SMYTH, H S. P SLEIGHT and G W PICKERING Reflex regulati n of arterial pressure during sl... quantitative method of assessi g baroreflex sensitivity *Circulat Res*. 1969 24 109–1 1

THANT M., Y YAMORI nd K. OKAMOTO, Baroreceptor function revealed by acute aortic ... in spontaneously hypertensive rats. *Jap. Circulat J* 1969 33 501 507

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Rate-dependent Myogenic Response of Vascular Smooth Muscle during Imposed Changes in Length and Force

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Abstract

SSON, S. B., B. JOHANSSON and S. MELLANDER. *Rate-dependent myogenic response of vascular smooth muscle during imposed changes in length and force.* Acta physiol. scand. 77 99 183–189

study of electrical and mechanical responses to stretch in isolated vascular smooth muscle revealed cut and graded influence of the rate of change in length, dL/dt (Johansson and Mellander, 1975) myogenic "dynamic" response at high rates of stretch was much more pronounced than the "static" e to constant, increased (40) length. In this previous study the mechanical responses were d as active force. In view of the fact that myogenic reactions in vivo must be associated th changes d caliber was considered of interest to investigate the responses to stretch also in smooth muscle ing active shortening. In the present study as in the one referred to above, electrical and mechanical ity in the isolated rat portal vein was studied by the sucrose gap method. However in the present ents the mechanical responses were recorded not only as active force but also as active shortening, t possible comparison of the myogenic responses under these two types of smooth muscle contrac Dynamic passive stretch was found to be associated th marked increases in spike discharge and ical activity under both these experimental conditions and the quantitative relation between spike rge and rate of passive stretch (or shortening) as similar. Thus, active shortening of the smooth did not interfere th the ability of the vessel to respond myogenically to passive stretch. A further s of the results suggested that, for the preparations as whole, the dynamic excitatory response was closely related to the rate of change of passive force, dP/dt, than to dL/dt

ular smooth muscle in resistance and precapillary sphincter vessels is sensitive to stretch responds to an increase in transmural pressure with enhancement of contractile activity kind of response is the background of myogenic reactions in the peripheral circulation mplified by myogenic autoregulation of blood flow and capillary hydrostatic pressure ref see g Folkow 1964 Mellander and Johansson 1968 Johnson 1974). Recent k on the vascular bed of skeletal muscle has indicated that the local vascular responses rise in transmural pressure are much more vigorous during the dynamic phase of changpressure than during sustained static pressure elevation (Mellander Lundvall and Grände 4, Grände, Lundvall and Mellander 1976), suggesting that the myogenic reaction involves a rate-sensitive element.

The cellular mechanisms behind such dynamic myogenic reactions were elucidated in a ent in itro study of electrical and mechanical activity in isolated vascular smooth muscle

Folkow B. M Hallbäck, Y Lundgren, R. Sivertsson and L. Weiss, Importance of adaptive ch in vascular design for establishment of primary hypertension, studied in man and in spontaneously h tensive rats. *Circulat Res* 1973 *32–33* Suppl. I I 2–I 13.

Fujishima, M T Sugi, Y Morotomi and T Omae, Effects of bilateral carotid artery ligation o lactate and pyruvate concentrations in normotensive and spontaneously hypertensive rats *Jap* *6* 62–66.

Goldberg A. L., Work-induced growth of skeletal muscle in normal and hypophysectomized rats *J Physiol.* 1967 *213* 1193–1198

Gribbin, B T G Pickering, P Sleight and R Peto, Effect of age and high blood pressure on reflex sensitivity in man. *Circulat Res.* 1971 *29* 424–431

Hilgenberg F Neurohistologic studies of the carotid sinus baroreceptors in hypertension *Baro and Hypertension* Ed P Kezdi Pergamon Press. New York. 1967 pp 293–296

Hilton, S. M Inhibition of baroreceptor reflexes on hypothalamic stimulation *J Physiol.* (Lond.) *165* 56–57

Kezdi, P Resetting of the carotid sinus in experimental renal hypertension. *Baroreceptors and Hypertension*, Ed. P Kezdi Pergamon Press. New York 1967 pp. 301 306.

Kirchheim, H R. Systemic arterial baroreceptor reflexes. *Physiol Rev* 1976. *56* 100–176

Korner, P I M J West J Shaw and J B. Uther, "Steady-state properties of the baroreceptor rate reflex in essential hypertension in man. *Clin. exp Pharmacol. Physiol* 1974 *1* 65–76.

Krieger E. M Neurogenic hypertension i the rat *Circulat Re* 1964 *15* 511 521

Krieger, E. M Time-course of baroreceptor resetting in acute hypertension. *Amer J Physiol* 1970 486–490.

Kylstra, P and B Lisander, Differentiated interaction between the hypothalamic defence area and receptor reflexes. *A ta physiol scand* 1970 *78* 386–392.

Lundgren Y M Hallbäck, L. Weiss and B. Folkow Rate and extent of adaptive cardiac changes in rats during experimental renal hypertension. *Act physiol. scand.* 1974. *91* 103–115

McCubbin, J W Carotid sinus participation in experimental renal hypertension. *Circulation* 791–797

McCubbin, J W J H Green and I H Page, Baroreceptor function in chronic renal hypertension *Circulat Res.* 1956. *4* 205 210.

Nosaka, S and K Okamoto Modified characteristics of the aortic baroreceptor activities in the spontaneously hypertensive rat. *Jap Circulat J* 1970. *34* 685–693

Nosaka, S. and S. C. Wang Carotid sinus baroreceptor functions in the spontaneously hypertensive *Amer J Physiol* 1972. *222* 1079–1084

Rees, P M Electron microscopical observations on the architecture of the carotid arterial wall special reference to the sinus portion. *J Anat.* (Lond.). 1968 *103* 35–47

Salgado H C. and E. M Krieger Reversibility of baroreceptor adaptation in chronic hypertension *Clin. Sci. Mol. Med* 1973 *45* 123–126.

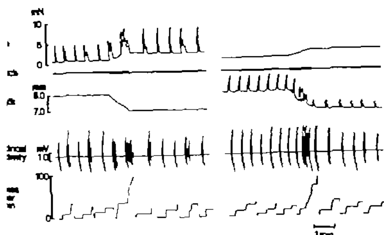
Sapru H. N and S C. Wang, Modification of aortic baroreceptor resetting in the spontaneously hypertensive rat. *Amer J Physiol* 1976. *230*. 664–674.

Sleight P B Gribbin nd T G Pickering Baroreflex sensitivity in normal and hypertensive man effect of beta-adrenergic blockade on reflex sensitivity *Postgrad med J* 1971 *47* 79–83.

Sleight P J L. Robinson D E. Brooks and P M Rees, Carotid baroreceptor resetting in the hypertensive dog. *Clin. Sci. Mol. Med.* 1975 *48* 261 263

Smyth H S., P Sleight and G W Pickering Reflex regulatio of arterial pressure during sleep in a quantitative method of assessing baroreflex sensitivity *Circulat Res.* 1969. *24* 109–121

Thant M Y Yamori nd K. Okamoto, Baroreceptor functio revealed by acut sinoaortic denervation in spontaneously hypertensive rats. *Jap Circulat J* 1969 *33* 501 507



...ct of dynamic and static passive stretch on mechanical and extracellularly recorded electrical ...he isolated rat portal vein. In the left panel the muscle was stretched by increasing the length by ...stant rate. In the right panel the muscle was stretched to the same final length and passive force, ...y increasing the load at constant rate

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(Johansson and Mellander 1975) These authors found pronounced and graded [illegible] responses to different rates of length changes in preparations of rat portal vein, exc[illegible] effects being observed in response to positive, and inhibitory effects to negative, [illegible] dL/dt For instance spike frequency increased to about 300 per cent of control [illegible] rates at or above 1.5 per cent of the muscle length per sec. A static increase in length [illegible] per cent led to only some 15 per cent increase in spike activity The electrical respon[illegible] associated with enhanced mechanical activity measured as contractile force. In view of [illegible] fact that myogenic reactions in vivo must lead to changes in vessel caliber in order to [illegible] hemodynamic parameters, such as resistance to flow it was considered of importance [illegible] investigate the electrical and mechanical responses of the smooth muscle also under c[illegible] tions which would permit active shortening. The present study is an extension of the [illegible] work on the isolated portal vein to encompass such mechanical conditions.

A preliminary report on the present study has been published (Sigurdsson, Joh[illegible] and Mellander 1976)

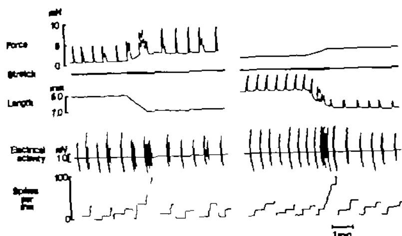
Methods

The portal vein of Sprague-Dawley rats (250–300 g) was dissected [illegible]nd mounted in the sucrose-gap [illegible] tus as described earlier (J[illegible]hansson [illegible]nd Mellander 1975). The active part of the preparation, 5 to 7 [illegible] length, was co[illegible]ti[illegible]uously superfused with standard Krebs sol[illegible]tion of the following composition [illegible] NaCl 122 KCl 4 73 N[illegible] HCO_3 15 5 KH[illegible]PO[illegible] 1 19 $MgCl_2$ 1 19 $CaCl_2$ 2.49 glucose 11.5 and [illegible] versenate 0 026 The sol[illegible]tion was bubbled with 4% CO_2 in O_2 giving a pH of 7.3–7 4 and the temp [illegible] was kept [illegible]t 37°C. Electrical activity was studied with the sucrose gap technique (see e g Burnst[illegible] Straub 1958). Electronic cou[illegible]ting [illegible]f the number of spikes was performed as in the earlier study (Joh[illegible] and Mellander 1975).

After the muscle had accommodated fo[illegible] at least 1 h at a low preload of about 0.7 mN [illegible] to passive stretch and shortening [illegible]t graded rates. These changes in length were produced in two [illegible] ways and two different techniques were used fo[illegible] recording the active mechanical responses. The exp[illegible] tal proced[illegible]res of the first type were as [illegible] the previous study (Johansson [illegible]nd Mellander 1975) in [illegible] muscle was connected to a force transducer (Grass FTO3) which could be raised and lowered at grad[illegible] by means of [illegible] micrometer screw The muscle was thereby exposed to changes in length at selected [illegible] values of dL/dt and the contractile responses were recorded as development of active force. [illegible] the [illegible] type of experimental procedure the muscle was connected t[illegible] an isotonic lever supplied with a photo [illegible] device which permitted recording of passive and active changes of muscle length. The load on t[illegible] could be varied in a conti[illegible]uous and co[illegible]trolled manner [illegible] a [illegible]piral spring connected to the le[illegible] With this arrangement the passive f[illegible]rce [illegible]pplied to the muscle co[illegible]ld be altered at selected constant [illegible] dP/dt while the contractile responses were recorded as active shortening. It was possible t[illegible] shift from one to the other mode of recording without altering the control situation with regard t[illegible] passi[illegible] and preload It should be noted that the two proced[illegible]res implied differences in time course of passi[illegible] and dP/dt due to the non-linear length–tension relation of the resting muscle. For instance, during [illegible] at constant dL/dt, there was [illegible] gradual increase in dP/dt whereas a [illegible]tretch at constant dP/dt [illegible] with [illegible] decreasing dL/dt (see further below)

Results

Fig. 1 shows original recordings of electrical and mechanical responses of the portal [illegible] passive stretch. The left panel is representative of the first type of experiment in wh[illegible] muscle was subjected to changes in length at constant dL/dt and developed contracti[illegible] without active changes in length. The panel shows from top to bottom the output fr[illegible]



Effect of dynamic and static passive stretch on mechanical and extracellularly recorded electrical y in the isolated rat portal vein. In the left panel the muscle was stretched by increasing the length by a constant rate. In the right panel the muscle was stretched to the same final length and passive force, ew by increasing the load at constant rate

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The right panel of Fig. 1 shows the results obtained in the same muscle under the second pe of experimental conditions where the muscle shortened actively during the phasic ntractions as shown in the length recording. Mean spike activity in the control period as now 27 spikes/min. The muscle was stretched to the same final length and passive force in the left panel but in this case the stretch was applied by increasing the load on the lever a constant dP/dt by means of the spiral spring. The continuous change in force could not e directly recorded under these conditions but was deduced from the calibration of the pring and is indicated t the top of the panel. It is evident that this type of passive stretch f the portal vein also led t a significant dynamic excitatory response, spike discharge ncreasing to 90 spikes/min despite the fact that the muscle could now counteract the passive lengthening by active shortening. At the later static stage of increased length and passive force, spike discharge was again only little changed compared t control activity

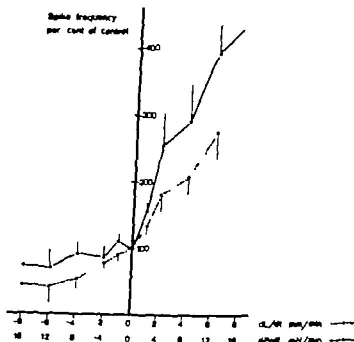
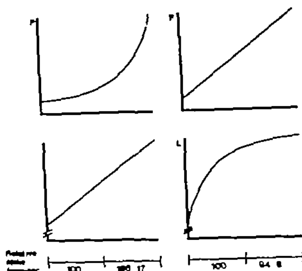


Fig. 2. Electrical response (mean ± S.E.) of the isolated rat portal vein to dynamic changes in length or passive force at rates ranging from − 8 to + 8 mm/min or − 16 to + 16 mN/min, respectively. Spike rate at control length was set to 100 %. The values for dL/dt are the absolute rates of length change applied in the experiments, the figures for dP/dt are given as mean values since passive compliance varied between different muscles.

The changes in spike discharge during the dynamic stretch shown in the left panel of Fig. 1 confirm the results of the earlier study (Johansson and Mellander 1975). The observations illustrated by the right panel of Fig. 1 indicate that passive stretch of the vascular smooth muscle is associated with a dynamic enhancement of spike activity also under conditions when active shortening can occur. Experiments were performed on a total of 7 portal vein preparations alternating repetitively throughout the individual experiment, between the two procedures for recording of mechanical responses and application of constant dL/dt and dP/dt, respectively. The total increase in length was always about 40 per cent of the muscle length prevailing at the low starting preload, but the rates of the changes in length and force were varied.

Fig. 2 summarizes the effects on spike activity obtained during the dynamic phases of stretch and shortening as a function of dL/dt or dP/dt in the two types of experimental procedure. The changes in spike activity are given as a percentage of the average frequency prevailing in the control period preceding each stretching of the muscle. Increases and decreases in spike activity with positive and negative dL/dt are qualitatively and quantitatively in agreement with the results reported in the earlier study. Fig. 2 shows that similar variations in spike discharge occurred when the muscles were passively stretched and shortened by application of positive and negative dP/dt under conditions where active shortening could take place. If anything, there is a relatively greater enhancement of spike discharge during stretch under these latter circumstances.



3 Schematic illustration showing how the rate of change in passive force increases when the muscle ... increased at constant rate (left diagrams, cf left panel of Fig. 1) and how the rate of change ... length decreases ... passive force is increased at constant rate (right diagrams, cf right panel of ... 1). At the bottom ... shown how the stretch period was divided into two halves. The spike activity in first half was set to 100. The number of spikes in the later half period was significantly greater when ... it was increasing during the stretch period (left diagrams) but not much changed when dP/dt ... constant and dL/dt decreasing during the stretch period (right diagrams).

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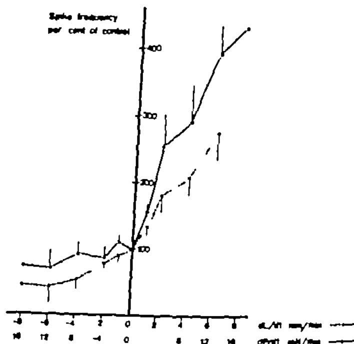
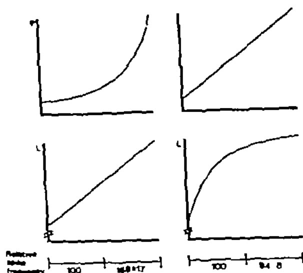


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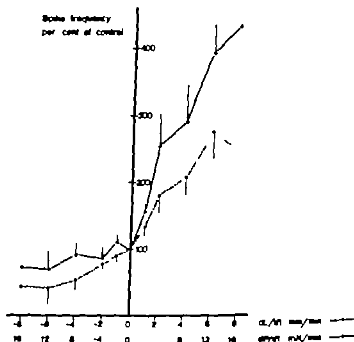


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References

E and Ch Wehs, Rate sensitivity of the mechanism of pressure change of vascular resistance. ract 1969 5 241–247

rock, G. and R. W. Straub, A method for studying the effects of ions and drugs on the resting and n potentials in smooth muscle with external electrodes. J Physiol. (Lond.) 1958, 140, 156–167

s. B Description of the myogenic hypothesis Circulat Res 1964 15 (suppl. I) I 279–285

n, P-O. L. Lundvall and S. Mellander, Evidence for rate-sensitive regulatory mechanism in ... microvascular control Acta physiol. scand. 1977 99 431–447

..., R. and S. Mellander, Static and dynamic components in the vascular myogenic response to ... changes in length as revealed by electrical and mechanical recordings from the rat portal vein. ... Res 1975 36. 76–81.

on, P C., The microcirculation, and local and humoral control of the circulation In Guyton and ... (eds), MTP International review of science Cardiovascular Physiology I p. 163–195. Butterworth, ... University Park Press, Baltimore. 1974

... S and B Johansson, Control of resistance, exchange, and capacitance functions in the peripheral circulation Pharmacol. Rev. 1968 20 117–196.

... S J Lundvall and P O Grände, Evidence for dynamic component in the myogenic ... of blood vessel tone in vivo. Acta physiol. scand. 1976. 96 40A–41A.

..., S B B Johansson and S. Mellander, Dynamic myogenic responses of the isolated rat portal ... to passive changes in length under isometric and isotonic conditions. Acta physiol scand. 1976 96.

...ll, T A biophysical analysis of mechano-electrical transduction. In Loewenstein (ed), Handbook ... sensory physiology I p 295 Springer-Verlag, Berlin. 1971

Discussion

Autoregulatory maintenance of constant blood flow in response to variations in pressure is a more or less prominent characteristic of most vascular beds. This kind of responsiveness requires, as an example, that a rise in arterial pressure elicits a correspon increase in resistance to flow by vasoconstriction. The myogenic theory of autoregula proposes that the transmural pressure itself creates the stimulus for enhancement of vas smooth muscle tone. Since vessel caliber must become reduced in the actual examp is evident that the sensing element involved in the myogenic autoregulatory reaction can be a simple length receptor responding to overall increases in vascular circumference. T kind of reasoning may apply also when the sensor responds to the rate of rise in pres rather than to the level of elevated pressure.

In view of such considerations it was important to find out whether the dynamic ste response, demonstrated in isolated myogenically active vascular muscle (Johansson & Mellander 1975) would remain or whether it would become impaired if the muscle co counteract the applied stretch by active contractile shortening. Fig. 1 showed that the elec physiological response to stretch was about the same when the muscle could shorten (ri panel) as when contraction manifested itself purely as force (left panel). The diagram Fig. 2 indicated that the relationships between spike frequency and rate of stretch or short ing are quite similar under the two conditions of mechanical response. It is evident there that active shortening neither abolished nor reduced the excitatory action of passive dyn stretch.

The contractile responses which accompany the changes in spike discharge deserve spe comments. The time average of contractile force during dynamic stretch (left panel of Fig increased as a result both of the enhancement of spike discharge and as a consequence of shift to a more favourable section of the length-active tension curve as discussed in the earl paper (Johansson and Mellander 1975) The enhancement of spike discharge and the crease in load will affect active contractile shortening in opposite directions. As indica by the right panel of Fig. 1 the increased electrical activity during stretch acts to incre the time average of active shortening but this effect is curtailed by the increasing load wh reduces the amplitude of the individual contractions. It appears from this recording that t excitatory response to stretch would be entirely insufficient to accomplish "autoregulato as the contractile shortening fails even to neutralize the passive lengthening. However t important points should be considered in this context First it should be realized that t relative magnitudes of passive lengthening and active shortening can be quite different greater preloads than those applied here In order to obtain sufficiently long periods stretch over which measurements could be made it was necessary to start at a minimal pr load in the present experiments, and in this situation the parallel elastic element of the musc is very distensible so that passive lengthening becomes quite large. Active shortening fai to compensate for this lengthening despite the appreciable excitatory membrane respons Intact vascular beds also fail to autoregulate at low arterial pressures, *i.e.* in the low range preloads on the vascular walls. The second point to be considered when comparing th right panel of Fig. 1 with the situation in the intact circulation is the law of Lapla

facilitate shortening in a cylindrical vessel while there is no equivalent to this mech- n in the present in vitro experiments. Therefore the results of the present study seem patible with findings of myogenic autoregulation in vivo in response to both dynamic static stimuli (Basar and Weiss 1969, Grände *et al.* 1976). The further analysis of our illustrated by Fig. 3 above indicated that the excitatory response to dynamic stretch more closely related to dP/dt than to dL/dt when considered for the preparation as a le. The exact implication of this finding with respect to the nature of the sensing element its localization in relation to the contractile units cannot be defined at present. It is of rest to note, however, that work on sensory mechanoreceptors have indicated that in e systems it is the ensuing change in tension rather than the actual length displace t which acts as the primary stimulus (Teorell 1971).

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References

AR, E. and CH. WEISS, Rate sensitivity of the mechanism of pressure change of vascular resistance. Pflügers Arch. 1969. 5. 241–247.

NSTOCK, G. and R. W. STRAUB, A method for studying the effects of ions and drugs on the resting and action potentials in smooth muscle with external electrodes. *J. Physiol.* (Lond.) 1958. *140*. 156–167.

LOW, B. Description of the myogenic hypothesis. *Circulat. Res.* 1964. *15* (suppl. I): I-279–285.

NDE, P.-O., J. LUNDVALL and S. MELLANDER, Evidence for a rate-sensitive regulatory mechanism in myogenic microvascular control. *Acta physiol. scand.* 1977. *99*. 432–447.

ANSSON, B. and S. MELLANDER, Static and dynamic components in the vascular myogenic response to passive changes in length as revealed by electrical and mechanical recordings from the rat portal vein. *Circulat. Res.* 1975. *36*. 76–83.

HNSON, P. C. The microcirculation, and local and humoral control of the circulation. *In*: Guyton and Jones (eds.), *MTP International review of science. Cardiovascular Physiology. 1*. p. 163–195. Butterworth, London. University Park Press, Baltimore. 1974.

LLANDER, S. and B. JOHANSSON, Control of resistance, exchange, and capacitance functions in the peripheral circulation. *Pharmacol. Rev.* 1968. *20*. 117–196.

MLLANDER, S., J. LUNDVALL and P.-O. GRÄNDE, Evidence for a dynamic component in the myogenic control of blood vessel tone in vivo. *Acta physiol. scand.* 1976. *96*. 40A–41A.

SJÖBLOM, S., B. JOHANSSON and S. MELLANDER, Dynamic myogenic responses of the isolated rat portal vein to passive changes in length under isometric and isotonic conditions. *Acta physiol. scand.* 1976. *96*. 17A.

TEORELL, T. A biophysical analysis of mechano-electrical transduction. *In*: Loewenstein (ed.), *Handbook of sensory physiology. 1*. p. 295. Springer Verlag, Berlin. 1971.

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Autoregulatory maintenance of constant blood flow in response to variations in pressure is a more or less prominent characteristic of most vascular beds. This kind of re-sponsiveness requires, as an example, that a rise in arterial pressure elicits a correspond-increase in resistance to flow by vasoconstriction. The myogenic theory of autoregula-proposes that the transmural pressure itself creates the stimulus for enhancement of smooth muscle tone. Since vessel caliber must become reduced in the actual example, it is evident that the sensing element involved in the myogenic autoregulatory reaction can-be a simple length receptor responding to overall increases in vascular circumference. T kind of reasoning may apply also when the sensor responds to the rate of rise in pres-rather than to the level of elevated pressure.

In view of such considerations it was important to find out whether the dynamic spec-response, demonstrated in isolated myogenically active vascular muscle (Johansson and Mellander 1975) would remain or whether it would become impaired if the muscle co-counteract the applied stretch by active contractile shortening. Fig. 1 showed that the elec-physiological response to stretch was about the same when the muscle could shorten (right panel) as when contraction manifested itself purely as force (left panel). The diagram i Fig. 2 indicated that the relationships between spike frequency and rate of stretch or short-ing are quite similar under the two conditions of mechanical response. It is evident there-that active shortening neither abolished nor reduced the excitatory action of passive dyn-stretch.

The contractile responses which accompany the changes in spike discharge deserve speci-comments. The time average of contractile force during dynamic stretch (left panel of Fig. 1 increased as a result both of the enhancement of spike discharge and as a consequence of the shift to a more favourable section of the length-active tension curve as discussed in the earlie-paper (Johansson and Mellander 1975). The enhancement of spike discharge and the in-crease in load will affect active contractile shortening in opposite directions. As indicated by the right panel of Fig. 1 the increased electrical activity during stretch acts to increase the time average of active shortening but this effect is curtailed by the increasing load which reduces the amplitude of the individual contractions. It appears from this recording that the excitatory response to stretch would be entirely insufficient to accomplish "autoregulation" as the contractile shortening fails even to neutralize the passive lengthening. However two important points should be considered in this context. First, it should be realized that the relative magnitudes of passive lengthening and active shortening can be quite different at greater preloads than those applied here. In order to obtain sufficiently long periods of stretch over which measurements could be made it was necessary to start at a minimal pre-load in the present experiments, and in this situation the parallel elastic element of the muscle is very distensible so that passive lengthening becomes quite large. Active shortening fails to compensate for this lengthening despite the appreciable excitatory membrane response. Intact vascular beds also fail to autoregulate at low arterial pressures, *i.e.* in the low range of preloads on the vascular walls. The second point to be considered when comparing the right panel of Fig. 1 with the situation in the intact circulation is the law of Laplace

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The findings summarized above suggest that α-receptor induced relaxation of the intestine ...pends on an action on the intramural cholinergic neurons and/or on its nerve endings. ...ey do not exclude the possibility however that inhibitory α-adrenoceptors located in the ...smooth muscle cells may contribute to the relaxation. Several investigators have reported ...at relaxation mediated by -adrenoceptors has been resistant to blockade by atropine or ...tetrodotoxin (Lee 1970, Bowman and Hall 1970). Lee (1970) observed that after blockade ...of β-adrenoceptors, epinephrine was more potent, in relaxing guinea pig circular ileum ...ntracted by histamine, in the absence than in the presence of atropine. H therefore ...ggested that the organ was relaxed by the inhibition of α-adrenoceptors located both in ...smooth muscle cells and in cholinergic neurons.

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When studying relaxing mechanisms having neuronal or muscular point of action there are some theoretical and methodological problems which have to be taken int account. A muscle contracted as a result of activation of neural elements in the intestine may be relaxed by an inhibitory mechanism located either in the neurons or in the smooth muscle cells of the organ. If the preparation is contracted by direct activation of the smooth muscle cells,

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From the Department of Pharmacology Linköping University Sweden

Localization of Adrenergic Receptors in Guinea Pig Ileum and Rabbit Jejunum to Cholinergic Neurons and to Smooth Muscle Cells

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Abstract

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The localization of adrenergic receptors mediating a relaxing action was investigated in innervated and denervated longitudinal muscle strips from guinea pig ileum and rabbit jejunum. Denervated preparations were contracted by drugs that had a direct effect on smooth muscle cells, such as acetylcholine and histamine, but not by stimuli acting on cholinergic neurons, such as electrical field stimulation or nicotine. After blockade of β-adrenoceptors, norepinephrine relaxed the innervated guinea pig ileum contracted by electrical field stimulation, by stimulating α-adrenoceptors. Norepinephrine in low concentrations did not relax denervated preparations contracted by agents acting directly on smooth muscle. In high concentrations, it relaxed denervated preparations by a nonadrenergic mechanism, resistant to α- and/or β-receptor blockade, but which was also activated by 1-(3 4-dihydroxyphenyl) ethanol. Phenylephrine only had an agonistic effect on the electrically stimulated innervated preparation and did not relax the denervated one. The denervated rabbit intestine contracted by acetylcholine was relaxed by norepinephrine and phenylephrine by stimulation of α-adrenoceptors. In the innervated preparations both drugs were more effective in inhibiting contractions induced by electrical field stimulation or eserine than those induced by exogenous acetylcholine. Both the denervated guinea pig and rabbit intestine were relaxed by stimulation of β-adrenoceptors. It is suggested that in the guinea pig ileum α-adrenoceptors mediating relaxation are located only in cholinergic neurons, whereas in rabbit jejunum they are located both in these neurons and in the smooth muscle cells. Beta-adrenoceptors are located in the smooth muscle cells of both organs.

The relaxing action of catecholamines in the intestine of many mammalian species is mediated by adrenergic α- and β-receptors (Ahlquist and Levy 1959 Furchgott 19[illegible] Bucknell and Whitney 1964 Andersson and Mohme Lundholm 1969 Bowman and H[illegible] 1970). The biochemical and biophysical events associated with the stimulation of these receptors differ in many respects (Jenkinson and Morton 1967 Brody and Diamond 19[illegible] Bülbring and Tomita 1969 Andersson and Mohme Lundholm 1970, Andersson 19[illegible] Bülbring 1973). It has been suggested that the relaxing effect mediated by α-adrenoceptors in guinea pig longitudinal ileum is due to an inhibition of cholinergic neurons innervating the intestine while the β-adrenoceptor-mediated effect is elicited by a direct action on the

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Histological examination of methylene blue stained preparations. The innervated preparations showed a dense network of dark-blue stained nervous lements (Fig. 1 A). In the microscope ganglia ...th several ganglionic cells were seen lying on the surface of the smooth muscle. Interconnecting nerve bundles were sometimes present between the ganglia. When the longitudinal muscle was denervated, as described under Methods (p. 192) the *proximal parts* usually contained the nervous plexus and often some circular muscle fibres. I the rest of the preparation the nervous plexus disappeared in a wedge-shaped fashion so that the distal parts became completely free from nervous elements (Fig. 1 B). In the embedded denervated preparations no nervous elements at all could be identified.

Contractile responses of innervated and denervated preparations. The innervated strips were contracted by acetylcholine and histamine which probably acted both on neural and muscular receptors. The preparations were also contracted by eserine and nicotine. The contractile effect of nicotine was inhibited by atropine (3.5 10− M) (Fig. 2 A, 1 5). In th...

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Methods

Male guinea pigs and rabbits of either sex were killed by a blow on the neck The small inte moved, the parts adjacent to the pylorus and colon being discarded, and dissected free fro The longitudinal muscle layers were removed from the circula muscle by different technique either innervated or denervated longitudinal muscle strips.

Innervated preparations

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with no neural participation, the muscle can only be relaxed by a direct effect on cells There are few drugs, if any that contract intestinal smooth muscle selectively w[illegible] any effect on its neurons. Drugs such as acetylcholine angiotensin, serotonin and ions such as barium and potassium, contract guinea pig ileum and other [illegible] parations by stimulating both cholinergic neurons and muscle cells (Ambache and L[illegible] 1955 Paton and Vane 1963 Paton and Zar 1968) This problem may be overcome by [illegible] a drug such as atropine to block the cholinergic receptors. Such an approach has [illegible] advantages, however Firstly the introduction of other drugs increases the risk of [illegible] unknown effects, *e.g.* interference with the receptor mechanism under investigation [illegible] condly the presence of an atropine resistant non-cholinergic excitatory neural [illegible] in guinea pig ileum has been suggested (Ambache and Freeman 1968). In this [illegible] main approach in the attempt to differentiate between α-adrenoceptors located at [illegible] and those located at muscle cells has been to compare the responses of innervat[illegible] denervated preparations.

Methods

Male guinea pigs and rabbits of either sex were killed by a blow on the neck. The small intestine [illegible] moved the parts adjacent to the pylorus and colon being discarded, and dissected free from [illegible] Th longitudinal muscle layers were removed from the circular muscle by different techniques to [illegible] either innervated or denervated longitudinal muscle strips.

Innervated preparations

Guinea pig The innervated specimens from the guinea pig were prepared as described by Rang [illegible] A piece of the intestine obtained appro imately 10 cm proximal to the ileocaecal sphincter [illegible] a glass rod The longitudinal muscle was removed t one end by stroking tangentially from the [illegible] border with a wisp of cotton wool. The rest of the muscle was then stripped off by pplying gentle [illegible] to the free end.

Rabbit Innervated specimens from the rabbit were prepared as described by Ambache (1954[illegible] that the jejunal part of the intestine was chosen instead of the ileum.

Denervated preparations

Guinea pig T obtai denervated preparations from the guinea pig the method described by Paton [illegible] (1968) was used. A longer piece of the i testine was thread on the glass rod and the longitudinal [illegible] was dissected free at one end as described above A lo g piece of longitudinal muscle was then p[illegible] In successful preparations about 25–50 cm of the ileum could be prepared in this way [illegible]ithout [illegible] breaking up into thin strands. Under a stereo microscope Auerbach plexus is readily identified [illegible] staining. It is often found that the distal parts of the preparation contain the plexus but that the [illegible] tissue disappears in wedge towards the proximal end so that this end becomes completely plexus[illegible] plexus-free part was selected and cut way from the plexus-retaini g portion.

Rabbit For denervating rabbit jejunum essentially the same technique was used as for guinea pi[illegible] A long piece of longitudinal muscle was obtained and inspected under the stereo microscope. The wedge-formed disappearance of the plexus seen i the guinea pig ileum was not observed in the intestine. Instead, ple us-free parts were often surrounded by innervated areas, or in some places [illegible] of innervated parts were left inside reas of denervated muscle The innervated and denervated are[illegible] clearly identifiable without staining and a denervated piece was carefully cut out with fine pair of [illegible]

The preparations were mounted i pecial holders, described by Lundholm and Mohme-L[illegible] (1966) and immersed in organ baths with capacity of 20 ml. The tension of the preparations was re[illegible] isometrically by means of a FT03 transducer nd a Grass polygraph. Electrically stimulated prep[illegible] were mounted in water-jacketed 4 ml perspex chamber In the walls of the hamber t o cm lon[illegible] num electrodes were mounted 1 cm apart parallel to the interposed preparation, permitting an [illegible] current to be passed through the Krebs solution and the muscle Stimulation was produced by [illegible]

pulses at 0.1 50 Hz either continuously or for 10 at intervals of one min with Grass S D9 sti-
and home constructed timer The strength of the stimulating field was always supramaximal.
reparations were kept in a Krebs solution of the following composition (in mM): NaCl 122 0, KCl
Cl_2 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 15 4, KH_2PO_4 1 2 and glucose 5.5, equilibrated with gas mixture of
and 95 O_2. The temperature of the Krebs solution was 37°C. The preparations were left in the
for 1 h before the experiments were started.

ne blue stained preparations

ted or denervated preparations were supravitally stained in methylene blue at 37°C. The staining
was composed of 0 3 g methylene blue and 9 0 g NaCl in 1 000 ml of water The preparations were
in Krebs solution and mounted on object glasses, and photographs were obtained immediately
Leitz Elmar (f 3 cm, 1 3 5). Some of the preparations were fixed in 8 % $(NH_4)_6MoO_{24}$, dehydrated
ethanol and ethanol and embedded in methyl metacrylate. Sections 10 µm thick were cut for micro-
examination.

cal methods

response curves were treated statistically Each of them was fitted into the logistic function

$$E = M \frac{A^P}{A^P + K^P}$$

ly the Taylor's theorem for non-linear least square regression analysis (Parker and Waud 1971).
formula, A is the concentration, K the ED_{50} value and M the maximal response to the drug. P is
nt which dependent on the slope of the dose-response curve. Values obtained experimentally are
as the arithmetic means ± S E Differences were tested by Student t-test for paired observations.
testing significance of ED_{50} values their logarithmic transformation (pD_2) was used.

following were used Acetylcholine chloride (Sigma), angiotensin amide (Ciba), atropine sulphate
), dibenamine hydrochloride (Astra), eserine salicylate (Merck), histamine chloride (ACO), indo-
ecan (Dumex), dl-isoprenaline sulphate (WB), carbachol chloride (ACO), morphine chloride
), l-norepinephrine bitartrate (Sigma), oxymetazoline chloride (Draco), pentolinium bitartrate
(May & Baker), 1-phenylephrine hydrochloride (Sigma), phentolamine methanesulphonate (Ciba), pro-
nolol chloride (ICI), sotalol hydrochloride (Mead–Johnson), tolazoline hydrochloride (Ciba–Geigy),
dotoxin (Sigma)

Results

gitudinal strips from guinea pig ileum

Histological examination of methylene blue stained preparations. The innervated preparations
wed a dense network of dark-blue stained nervous elements (Fig. 1 A). In the microscope
ight with several ganglionic cells were seen lying on the surface of the smooth muscle.
terconnecting nerve bundles were sometimes present between the ganglia. When the longi-
dinal muscle was denervated, as described under Methods (p 192) the proximal parts
ually contained the nervous plexus and often some circular muscle fibres. In the rest of the
reparation the nervous plexus disappeared in a wedge-shaped fashion so that the distal
rts became completely free from nervous elements (*Fig. 1 B*). In the embedded denervated
reparations no nervous elements at all could be identified.

Contractile responses of innervated and denervated preparations. The innervated strips
ere contracted by acetylcholine and histamine which probably acted both on neural and
muscular receptors. The preparations were also contracted by eserine and nicotine. The
contractile effect of nicotine was inhibited by atropine (3.5 10− M) (Fig. 2 A, 1–5). In the



Fig. 1 (A) Photomicrograph of an innervated strip of guinea pig ileum stained with methylene blue. Auerbach's plexus is seen dark-stained against the paler smooth muscle. At the margins of the preparation the plexus has been removed so that these parts appear plexus-free. Horizontal bar indicates 1 mm. (B) Denervated strip of guinea pig ileum stained with methylene blue. To the left, at the proximal end, a small area of nervous tissue is seen. In the rest of the preparation the longitudinal muscle is free from fibres and ganglia.

denervated strips histamine and acetylcholine elicited a contractile effect but eserine and nicotine did not (Fig. 2 B, 1–4)

Electrical field stimulation by (1 ms) single pulses at 0.1 Hz induced a twitch contraction at each pulse in the innervated preparations. The contractions were completely abolished by atropine (3.5×10 M) and by tetrodotoxin (3 10 M) (Fig. 3 A 1–3). Tetanic field stimulation (10 Hz for 10 s) produced stronger contractions which were reduced but

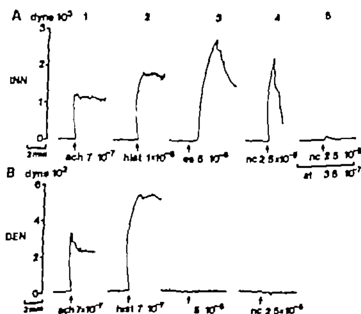
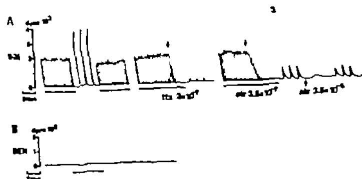


Fig. 2. (A) Responses of an innervated strip of guinea pig ileum. A 1–4 Acetylcholine 7 10⁻ M (ach), histamine 1 10⁻⁶ M (hist), eserine 5 10⁻⁶ M (es) and nicotine 2.5 10⁻⁶ M (nc), respectively. A 5 Nicotine 2.5 10⁻⁶ M (nc) in the presence of atropine 3.5 10⁻ M ( ) which had been added 10 min previously (B) Responses of a denervated strip of guinea pig ileum B 1–4 Acetylcholine 7 10⁻⁷ (ach), histamine 7 10⁻⁷ M (hist), eserine 5 10⁻⁶ M (es) and nicotine 2.5 10⁻⁶ M (nc), respectively.



) Responses of an innervated strip of guinea pig ileum. A 1 3 The horizontal bars indicate field stimulation at 0 1 Hz with 1 ms rectangular pulses. At the dots, field stimulation at 10 Hz The arrows indicate addition of tetrodotoxin 3 10⁻⁶ M (ttx) and atropine 3 5 10⁻⁷ – 3.5 (atr), respectively (B) Responses of denervated strip of guinea pig ileum. (Same preparation calibration as in experiment 2 B, 1–4). The horizontal bar indicates electrical field stimulation 0 1 Hz with 1 ms pulses. At the dots, field stimulation at 10 Hz for 10 s. At the asterisks, field at 50 Hz for 10 s.

ed by atropine (3.5 10⁻ 3.5 10⁻⁶ M). Tetrodotoxin almost completely inhibited ponse (Fig. 3 A, 1 3). In the denervated preparations both kinds of electrical stimula- re totally ineffective (Fig. 3 B). These observations indicate that on electrical stimula e innervated preparations were contracted by a cholinergic mechanism. At higher tion rates a non-cholinergic mechanism might also have contributed to the contrac

α-adrenoceptor mediated relaxation in innervated strips. In the innervated strip in β-adrenoceptors were blocked by sotalol (5 10⁻⁶ M), norepinephrine (3 10⁻⁶ M) ted the contractions elicited by electrical stimulation t 0.1 Hz to 92 ± 5% (n 6) 4 A, 1) This effect was almost completely blocked by tolazoline (3 10⁻⁵ M) (Fig. 4 A, hen the innervated strip was contracted by acetylcholine (7 10⁻⁸ M), the same con- tion of norepinephrine (3 10⁻⁶ M) relaxed the preparation to 52 ± 2% (n 5) (Fig.). Phenylephrine (1 10⁻ M) produced only minor and often transitory effects in the ically stimulated preparations (Fig. 4 A, 3). Following contraction by acetylcholine, ylephrine in concentrations of up to 5 10⁻⁴ M had a weak transitory relaxing action 4 B, 2) or none at all

ctile response in denervated strip To ascertain that the preparations were functionally rvated and that their contractile ability was not damaged, they were always tested with ne (5 10⁻⁶ M) and acetylcholine (7 10⁻ M). The mean tension induced by eserine e denervated preparations was only 1.6 ± 0 7 (= 9) of that induced by acetylcholine he same strip In the innervated strips, in contrast, the eserine contraction was 231.9 ± (n 5) of the mean tension caused by acetylcholine. When tested, the denervated parations invariably failed t respond to nicotine or tetanic field stimulation.

When the relaxing action of α-adrenoceptor-stimulation was tested in the denervated ps, these were pre-treated with sotalol (5 10⁻⁶ M) to block β-adrenoceptors and then tracted by acetylcholine (7 10⁻ M), carbachol (7 10⁻⁸ M), or histamine (1 10⁻⁶ M). epinephrine in concentration of 3 10⁻⁶ M, which relaxed the innervated strip, had



Fig 1 (A) Photomicrograph of an innervated strip of guinea pig ileum stained with methylene blue. Auerbach's plexus is seen dark-stained against the paler smooth muscle. At the margins of the preparation the plexus has been removed so that these parts appear plexus-free. Horizontal bar indicates 1 mm (B) Denervated strip of guinea pig ileum stained with methylene blue. To the left, at the proximal end, a small piece of nervous tissue is seen. In the rest of the preparation the longitudinal muscle is free from nerve cells and ganglia.

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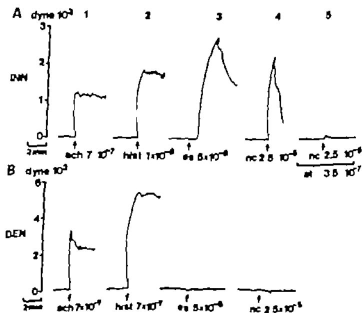


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5. (A) Responses of denervated strips of guinea pig ileum. Contraction of the preparations was maintained by carbachol 7×10^{-7} M, which had been added 10–15 min previously. Sotalol 5×10^{-6} M present in all experiments. A, 1: Norepinephrine 3×10^{-6} and 3×10^{-5} M (ne). A, 2: Norepinephrine 10^{-5} M (ne), after 30 min pretreatment with dibenamine 8×10^{-6} M (dba). A, 3: 1(3,4-dihydroxyphenyl)-...ol 3×10^{-6} M (dhpe). A, 4: Phenylephrine 5×10^{-6} M (phe). (B) Responses of denervated strip of ...ea pig ileum. Contraction of the preparation was maintained by histamine 7×10^{-8} M, which had ... added 10 min previously. B, 1: Isoproterenol 4×10^{-7} M (iso). B, 2: Isoproterenol 4×10^{-7} M (iso) ...e presence of sotalol 5×10^{-6} M (sot). B, 3: Isoproterenol 4×10^{-7} M (iso) in the presence of propranolol 10^{-6} M (prop)

...appearance observed in the guinea pig was not seen on denervation of the rabbit jejunum. ...tead, plexus-free parts were often surrounded by innervated areas, or innervated patches ...re sometimes left inside areas of denervated muscle (Fig. 6 B). This necessitated extra ...re in selection of denervated pieces for the experiments, no difficulty was encountered, ...wever since the innervated and denervated areas could be differentiated without staining. ...the embedded, denervated pieces, no nervous elements at all were observed.

Contractile responses of innervated and denervated preparations. Innervated longitudinal ...rips from rabbit jejunum showed spontaneous contractions which were regular or some...mes periodic. These contractions were unaffected or only slightly inhibited by atropine

Fig. 6. (A) Photomacrograph of an innervated strip of rabbit jejunum stained with methylene blue. Numerous ganglia with primary and secondary plexuses are seen dark-stained against the paler longitudinal smooth muscle. The horizontal bar indicates 1 mm. (B) Denervated preparation from rabbit jejunum stained with methylene blue. To the left, patches of innervated areas are seen. The rest of the preparation is free from visible nerves and ganglia.

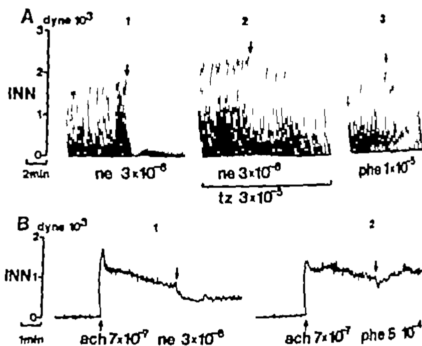


Fig. 4 (A) Responses of innervated strips of guinea pig ileum. The preparations were stimulated ele at 0.1 Hz All experiments were perf rmed in the presence of sotalol 5 10^{-6} M A 1 Norepi 3 10^{-6} M (ne). 2. Norepinephrine 3 10^{-6} M (ne) in the presence of tolazoline 3 10^{-5} M (tz Phenylephrine 1 10^{-5} M (phe). (B) Responses of n innervated strip of guinea pig ileum precon sotalol 5 10^{-6} M B 1 Acetylcholine 7 10^{-7} M (ach) and norepinephrine 3 10^{-6} M (ne). B, 2. choline 7×10^{-7} M (ach) and phenylephrine 5 10^{-4} M (phe).

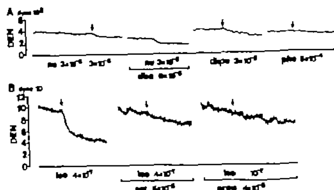
no effect on the β-adrenoceptor blocked denervated preparation In a higher con tion (3 10^{-4} M) norepinephrine induced moderate relaxation (Fig. 5 A, 1). This rela however was not inhibited by any of the α-adrenoceptor antagonists tested—dibe (4–8 10^{-4} M) (Fig. 5 A, 2), tolazoline (1 10^{-4} M) and phentolamine (3 10 -1 1(The effect was not influenced by propranolol (4 10^{-6} M) and neither was it inhibite the preparation had been incubated with indomethacin (1 10^{-4} M) for one hour The of norepinephrine (bi-distilled water) did not induce the effect but the norepin nucleus 1-(3 4-dihydroxyphenyl)-ethanol (3 10^{-3} M) had a similar relaxant effect (Fi 3) The relaxation induced by norepinephrine in this high concentration was therefor ably not due to the stimulation of adrenergic receptors.

Phenylephrine in concentrations up to 5 10^{-4} M did not relax the denervated p tions (Fig. 5 A 4).

The denervated strips were markedly relaxed by isoproterenol (4 10 M) a effect was inhibited both by sotalol (5 10^{-6} M) and by propranolol (4 10^{-6} M) (Fi 1–3)

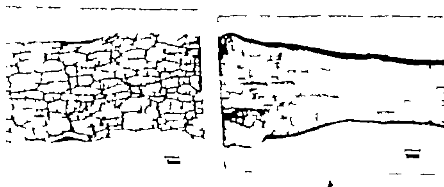
Longitudinal strips from rabbit jejunum

Histological examination of methylene blue stained preparations The innervated prepa showed a nervous plexus with an organization similar to that in guinea pig ileum (Fig The nervous network was somewhat more sparse than in the guinea pig and fewer a and nerve bundles were identified in the embedded preparations. The typical wedge-f



A) Responses of denervated strips of guinea pig ileum. Contraction of the preparations was main-
y carbachol 7 10^{-7} M, which had been added 10–15 min previously. Sotalol 5 10^{-6} M
ent in all experiments. A, 1 Norepinephrine 3 10^{-6} and 3 10^{-6} M (ne). A 2: Norepinephrine
M (ne), after 30 min pretreatment with dibenamine 8 10^{-6} M (diba). A, 3 1(3,4-dihydroxyphenyl)-
3 10^{-6} M (dopa). A 4 Phenylephrine 5 10^{-6} M (phe) (B) Responses of denervated strip of
pig ileum. Contraction of the preparation was maintained by histamine 7 10^{-6} M, which had
ded 10 min previously. B 1 Isoproterenol 4 10^{-7} M (iso). B 2. Isoproterenol 4 10^{-7} M (iso)
resence of sotalol 5 10^{-6} M (sot) B 3 Isoproterenol 4 10^{-7} M (iso) in the presence of propranolol
M (prop)

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. 6 (A) Photomicrograph of an innervated strip of rabbit jejunum stained with methylene blue. Numerous
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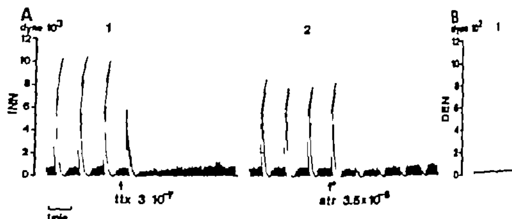


Fig. 7 (A) Response of an innervated strip of rabbit jejunum. At the dots, electrical field stimulation 10 Hz for 10 s. The arrows indicate addition of tetrodotoxin 3 10⁻⁷ M (ttx) and atropine 3.5 10⁻⁶ M (atr), respectively (B) Response of a denervated strip of rabbit jejunum. Same preparation ... calibration as in experiment 11 B, 1 2). The dots indicate electrical field stimulation at 10 Hz for 10 s.

(3.5 10 -3.5×10^{-6} M) or by tetrodotoxin (3 10 -3 10^{-6} M) (Fig. 7 A, 1 2). Electrical field stimulation at 1 to 10 Hz for 10 s with 1 ms pulses produced marked contractions which were usually followed by a period of inhibition. Atropine (3.5 10^{-7}–3.5 10^{-6}) inhibited the contractile but not the inhibitory response whereas tetrodotoxin (3 10 ...) inhibited both responses (Fig. 7 A, 1–2) The innervated preparation was contracted by acetylcholine (7 10 M) histamine (7 10^{-6} M) and eserine (5 10^{-6} M) (Fig. 8 A, 1–3).

Denervated rabbit preparations showed no or very little spontaneous activity; when it did occur it was weak and irregular The preparations failed to contract on tetanic field stimulation

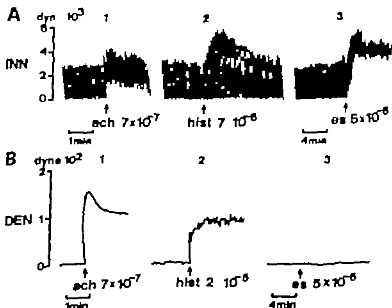
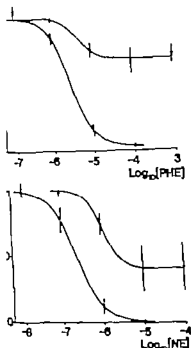


Fig. 8 (A) Responses of an innervated strip of rabbit jejunum. A 1 3: Acetylcholine 7 10⁻⁷ M (ach), histamine 7 10⁻⁶ M (hist) and eserine 5 10⁻⁶ M (es), respectively (B) Responses of denervated strip of rabbit jejunum. B 1 3 Acetylcholine 7 10⁻⁷ M (ach), histamine 2 10⁻⁵ M ... eserine 5 10⁻⁶ M



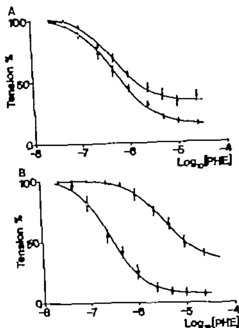


Fig. 9

Fig. 10

9 (A) Dose-response curves of the effect of phenylephrine (PHE) in the presence of propranolol 10^{-6} M on the innervated rabbit jejunum. Phenylephrine was added 30 before stimulation with either acetylcholine or an electric current. ● Contraction by acetylcholine 7×10^{-7} M. ○ Electrical field stimulation at 1 Hz for 10 each run. The continuous sigmoid curves represents the regression line of the logistic function to the experimental data. Each point represents the mean ± S.E. of five experiments. (B) A similar experiment to 9 A but norepinephrine (NE) was used instead of phenylephrine. ● Contraction elicited by acetylcholine 7×10^{-7} M. ○ Electrical field stimulation at 1 Hz for 10 each run (n = 6).

10 (A) Dose-response curves of the inhibitory effect of phenylephrine (PHE) on innervated strips of rabbit jejunum. Contraction of the preparations maintained by eserine 1×10^{-6} M. ● Contraction maintained by acetylcholine 1×10^{-6} M (n = 6). (B) A similar experiment to 10 A. ○ Contraction of the preparations maintained by eserine 6×10^{-7} M. ● Contraction by acetylcholine 1×10^{-6} M in the presence of propranolol 2×10^{-6} M (n = 6).

on (Fig. 7 B, 1 and 12, 3). Denervated preparations were contracted by acetylcholine ($7 \times 10^{-}$ M) and histamine (7×10^{-6} – 2×10^{-6} M) but responded only very slightly to eserine (1×10^{-6} M) (Fig. 8 B, 1, 3 and 12, 1, 2). Before any expts. were performed on the denervated preparations they were always tested with acetylcholine (7×10^{-7} M) and eserine (5×10^{-6} M). The contraction induced by eserine on denervated strips was 4.2 ± 0.9% (n = 12) of that induced by acetylcholine. The corresponding value in the innervated strips was 113 ± 3% (n = 5).

Alpha-adrenoceptor mediated relaxation in innervated strips. Dose-response curves of the inhibitory effects of norepinephrine and phenylephrine were obtained from innervated rabbit jejunal strips both during contraction with electrical field stimulation for 10 s at 1 Hz and after treatment with acetylcholine ($7 \times 10^{-}$ M) (Fig. 9 A, B). Propranolol (4×10^{-6} M)

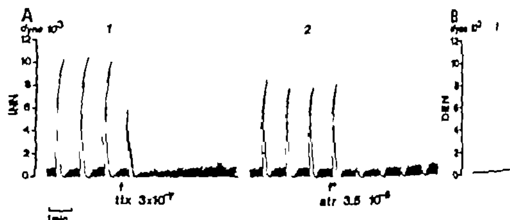


Fig. 7 (A) Response of an innervated strip of rabbit jejunum. At the dots, electrical field stimul... 10 Hz for 10 s. The arrows indicate addition of tetrodotoxin 3 10⁻ M (ttx) and atropine 3.5 ... (atr), respectively (B) Response of a denervated strip of rabbit jejunum. Same preparation with equal calibration as in experiment II B, 1). The dots indicate electrical field stimulation at 10 Hz for ...

(3.5 × 10 − 3.5 10⁻⁴ M) or by tetrodotoxin (3 10 ⁷ − 3 10⁻⁴ M) (Fig. 7 A, 1-2). Electrical field stimulation at 1 to 10 Hz for 10 s with 1 ms pulses produced marked contractions which were usually followed by a period of inhibition. Atropine (3.5 10⁻ 3.5 ...) inhibited the contractile but not the inhibitory response whereas tetrodotoxin (3 10 ...) inhibited both responses (Fig. 7 A, 1–2) The innervated preparation was contracted by acetylcholine (7 × 10 M) histamine (7 × 10⁻⁴ M) and eserine (5 10⁻⁴ M) (Fig. 8 A, 1-...

Denervated rabbit preparations showed no or very little spontaneous activity when it did occur it was weak and irregular The preparations failed to contract on tetanic field stim-

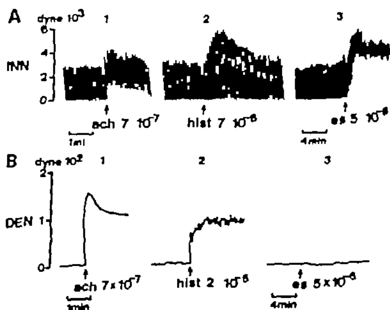
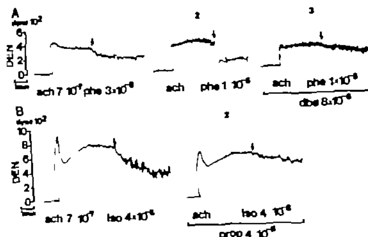


Fig. 8 (A) Responses of innervated strip of rabbit jejunum. A 1 3 Acetylcholine 7 10⁻⁷ M (ach) histamine 7 10⁻⁴ M (hist) and eserine 5 10⁻⁴ M (es), respectively (B) Responses of denervated strip of rabbit jejunum. B 1 3 Acetylcholine 7 10⁻ M (ach), histamine 2 10⁻⁴ M and eserine 5 10⁻⁴ M



1) Responses of denervated strip of rabbit jejunum. A 1–2. Acetylcholine 7 10⁻⁷ M (ach) phenylephrine 3 10⁻⁶ 1 10⁻⁶ M (phe) in the presence of sotalol 5 10⁻⁶ M. A; 3 Acetylcholine (ach) and phenylephrine 1 10⁻⁶ M (phe) in the presence of dibenamine 8 10⁻⁶ M (dbe) and 10⁻⁶ M (B) Responses of denervated strip of rabbit jejunum B 1 Acetylcholine 7 10⁻⁷ M isoproterenol 4 10⁻⁶ M (iso) B 2: Acetylcholine 7 10⁻ M (ach) and isoproterenol 4 10⁻⁶ M presence of propranolol 4 10⁻⁶ M (prop).

(7 10⁻⁶ M) relaxed the denervated preparation contracted by angiotensin (1–2), carbachol (7 10 M) (Fig. 12, 4–5) or acetylcholine (7 10⁻ M). If the prepara-s contracted by angiotensin the relaxation induced by norepinephrine was complete basal tone level (Fig. 12, 4) The relaxation induced by norepinephrine was blocked ntolamine (5 10⁻⁶ M) (Fig. 12, 6) or by dibenamine (8 10⁻⁶ M). After blocking ptors with sotalol (5 10⁻⁶ M) oxymetazoline (2 10⁻⁶ M) relaxed the denervated ation contracted by carbachol (7 10⁻ M). The relaxation induced by oxymetazoline ocked by dibenamine (8 10⁻⁶ M).

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Discussion

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rvation of longitudinal muscle strips from the guinea pig and rabbit intestine depends e anatomical features of these organs.

e fine structure of the guinea pig ileum has been thoroughly mapped out by electron oscopic investigations (Gabella 1972 a, b). In the ileum of this animal the region be n the longitudinal and circular muscle layers is occupied by the myenteric plexus, con-ng of ganglia and connecting strands of nerve fibres, together with interstitial cells and od vessels. I the circular muscle, there are numerous nerve fibres, which mainly run allel to the muscle fibres. The nerves contain varicose structures with several types of icles similar to those found in vesiculated nerve processes within the ganglia. At some

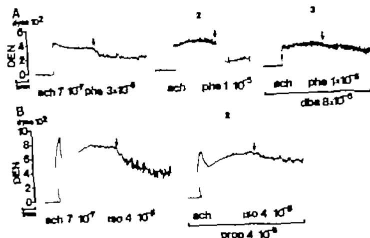
was present throughout the experiments in order to block β-adrenoceptors. The drugs added 30 s before the preparations was stimulated. The maximal inhibitory actions of [illegible] pinephrine (100.1 ± 0.3%) and phenylephrine (95.5 ± 1.7%) were significantly greater [illegible] the preparation was contracted by electrical stimulation than when it was contracted [illegible] acetylcholine (61.9 ± 14.0%, $p < 0.05$ and 37.3 ± 4.7%, $p < 0.001$ respectively). The [illegible] of the inhibitory action of norepinephrine during electrical stimulation was 3.1 [illegible] M but after contraction with acetylcholine it was significantly greater (2.0 ± 0.3 [illegible] $p < 0.02$). The corresponding ED_{50} values for phenylephrine were 2.8 ± 0.3 10^{-6} M and 1.7 × 10^{-6} M respectively ($p < 0.1$).

The differences in the potencies and efficacies of norepinephrine and phenylephrine [illegible] tween their inhibition of contractions induced by electrical stimulation and by exog[illegible] acetylcholine might indicate that the drugs were able to inhibit the release of acetylch[illegible] from cholinergic neurons in the preparations. To further investigate this question a com[illegible] son was set up to investigate how phenylephrine was able to relax the innervated pr[illegible] tion contracted by eserine (1 10^{-6} M) and by acetylcholine (1 10^{-6} M) (Fig. 10 A[illegible] maximal relaxation induced by phenylephrine was significantly greater after eserine co[illegible] tion (88.1 ± 1.9%) than after contraction by acetylcholine (67.2 ± 4.4%, p 0.01). The [illegible] ence was not large and the ED_{50} of eserine and acetylcholine were similar (5.4 ± 0.7 [illegible] and 5.6 ± 0.7 10 M respectively). Since the added acetylcholine might have stim[illegible] neurons mediating a contractile action, these experiments were repeated after prepa[illegible] contracted by acetylcholine had been pretreated by the ganglionic blocking agent [illegible] linium (2 10^{-6} M). The eserine concentration was lowered to 6 10 M in order to [illegible] a similar degree of contraction in the two series (Fig. 10 B). Under these conditio[illegible] ED_{50} of phenylephrine after contraction by eserine (3.3 ± 0.5 10 M) was signi[illegible] lower than after treatment with acetylcholine in the presence of pentolinium (3.6 [illegible] 10^{-6} M, $p < 0.001$). The maximal inhibition caused by phenylephrine was also signif[illegible] higher after treatment with eserine (92.8 ± 2.9%) than after the combination of acetylch[illegible] and pentolinium (70.3 ± 4.8%, $p < 0.02$).

During contraction by acetylcholine (1 10^{-6} M) the relaxing action of phenyleph[illegible] a concentration of up to 1 10 M was completely inhibited by dibenamine (8 10 [illegible] At a still higher concentration (2.5 10^{-4} M) phenylephrine had a weak relaxing [illegible] which was blocked by the combination of dibenamine (8 10^{-6} M) and sotalol (5 10 [illegible] The inhibitory effect of phenylephrine (1 10^{-5} M) during electrical stimulation was blocked by dibenamine (8 10^{-6} M).

Relaxant responses in denervated preparations. When the denervated preparation was [illegible] tracted by acetylcholine (7 10 M) it was relaxed by phenylephrine (3 10^{-4} 1 10 [illegible] (Fig. 11 A, 1-2). The relaxation was blocked by dibenamine (4-8 10^{-6} M) but not by [illegible] (5 10^{-6} M) (Fig. 11 A, 1-3). Phenylephrine also had a relaxing action in preparatio[illegible] tracted by carbachol (7 10 M), histamine (7 10^{-6} M) and angiotensin (1 10 [illegible] The relaxing action of phenylephrine was not affected by morphine (1-9 10^{-6} M) or [illegible] dotoxin (3 10 M). After contraction of the preparation by histamine or angiotens[illegible] relaxing effect of phenylephrine was not changed by atropine (3.5 10^{-6} M).

In the presence of sotalol (5 10^{-6} M) or propranolol (4 10 [illegible] 10^{-6} M) no[illegible]



(A) Responses of denervated strip of rabbit jejunum. A, 1 2 Acetylcholine 7 10⁻ M (ach) phenylephrine 3 10⁻⁶ 1 10⁻⁵ M (phe) in the presence of sotalol 5 10⁻⁶ M. A 3 Acetylcholine ⁷ M (ach) and phenylephrine 1 10⁻⁶ M (phe) in the presence of dibenamine 8 10⁻⁶ M (dba) and 5 10⁻⁶ M (B) Responses of denervated strip of rabbit jejunum. B 1 Acetylcholine 7 10⁻⁷ M nd isoproterenol 4 10⁻⁶ M (iso) B 2: Acetylcholine 7 10⁻⁷ M (ach) and isoproterenol 4 10⁻⁶ M i the presence of propranolol 4 10⁻⁶ M (prop)

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The differences in the potencies and efficacies of norepinephrine and phenylephrine between their inhibition of contractions induced by electrical stimulation and by exogenous acetylcholine might indicate that the drugs were able to inhibit the release of acetylcholine from cholinergic neurons in the preparations. To further investigate this question a comparison was set up to investigate how phenylephrine was able to relax the innervated preparation contracted by eserine (1 × 10^{-6} M) and by acetylcholine (1 × 10^{-6} M) (Fig. 10 A). The maximal relaxation induced by phenylephrine was significantly greater after eserine contraction (88.1 ± 1.9%) than after contraction by acetylcholine (67.2 ± 4.4%, $p < 0.01$). The difference was not large and the ED_{50} of eserine and acetylcholine were similar (5.4 ± 0.7 × 10^-[illegible] and 5.6 ± 0.7 × 10^-[illegible] M respectively). Since the added acetylcholine might have stimulated neurons mediating a contractile action, these experiments were repeated after preparations contracted by acetylcholine had been pretreated by the ganglionic blocking agent pentolinium (2 × 10^{-6} M). The eserine concentration was lowered to 6 × 10^-[illegible] M in order to obtain a similar degree of contraction in the two series (Fig. 10 B). Under these conditions the ED_{50} of phenylephrine after contraction by eserine (3.3 ± 0.5 × 10^-[illegible] M) was significantly lower than after treatment with acetylcholine in the presence of pentolinium (1.6 × 10^{-6} M, $p < 0.001$). The maximal inhibition caused by phenylephrine was also significantly higher after treatment with eserine (92.8 ± 2.9%) than after the combination of acetylcholine and pentolinium (70.3 ± 4.8 %, $p < 0.02$).

During contraction by acetylcholine (1 × 10^{-6} M) the relaxing action of phenylephrine in a concentration of up to 1 × 10^{-6} M was completely inhibited by dibenamine (8 × 10^-[illegible] M). At a still higher concentration (2.5 × 10^{-6} M) phenylephrine had a weak relaxing effect which was blocked by the combination of dibenamine (8 × 10^{-6} M) and sotalol (5 × 10^-[illegible] M). The inhibitory effect of phenylephrine (1 × 10^-[illegible] M) during electrical stimulation was blocked by dibenamine (8 × 10^{-6} M).

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In the presence of sotalol (5 × 10^-[illegible] M) or propranolol (4 × 10^-[illegible] – 1 × 10^{-6} M) non-

scopy using silver impregnation. Between the longitudinal and circular muscle are ganglia of Auerbach's plexus. Nerve fibres running in this region consist of the largest primary bundles dividing up into a finer secondary and tertiary plexus. From the tertiary plexus very fine nerve bundles together with blood capillaries and interstitial cells enter the muscle tissue and ramify between smooth muscle cells of both longitudinal and circular muscle. Richardson observed that when the circular muscle was stripped from the longitudinal muscle during the preparation of the tissue, the tertiary plexus was often damaged or completely removed from the longitudinal muscle.

In the methylene blue stained innervated rabbit jejunum used in this study ganglia and primary and secondary nerve bundles were observed, as in guinea pig ileum. The denervated strips lacked these nervous elements (Fig. 6 A, B). As with guinea pig ileum it could not be excluded that small nerves not visible in the stereo microscope remained in the preparation.

The innervated preparations showed a biphasic response on electrical stimulation i.e. a contraction followed by an inhibition of the spontaneous motility. Since the contractions were abolished by atropine they were probably mediated by acetylcholine released from the cholinergic neurons of the preparations. The inhibitory response to electrical stimulation was not affected by atropine but was probably of nervous origin, since it was abolished by tetrodotoxin. The denervated preparations did not respond to electrical field stimulation but were contracted by agents such as acetylcholine, carbachol and histamine which are considered to act directly on smooth muscle. In the denervated preparations eserine induced only a minute response in comparison with that resulting from acetylcholine. The innervated preparations on the other hand, responded by a similar degree of contraction to these two drugs.

Since the rabbit small intestine seems to be deeply innervated by small nerve fibres, clearly demonstrable in the electron microscope (Richardson 1958), it is probable that the denervation procedure used in this study leaves the intramuscular nerve endings intact while the ganglia and larger nerves—and probably the tertiary nerve plexus located between the longitudinal and circular muscle—are removed. The small response to eserine observed in the denervated preparations is probably due to a potentiation of the action of acetylcholine released from the intramuscular nerve endings left in the preparations. These nerves, however, were not able to induce contractions of the muscle on electrical stimulation at the same strength, frequency and duration of impulses that contracted the innervated preparations. The functional ability of the nerve endings left in the preparations was thus so impaired that they were unable to contract the preparation to any appreciable extent. Under such conditions a relaxing mechanism acting on the neurons could only affect the tone of the smooth muscle to a very limited degree.

Adrenergic receptors in longitudinal guinea pig ileum

The innervated strips from guinea pig ileum possessed α-receptors capable of inducing an inhibitory response. Norepinephrine in low concentrations inhibited electrical field stimulation at 0.1 Hz. The contractions following electrical stimulation at this frequency were probably mediated by the activation of cholinergic neurons, since they were abolished by atro-

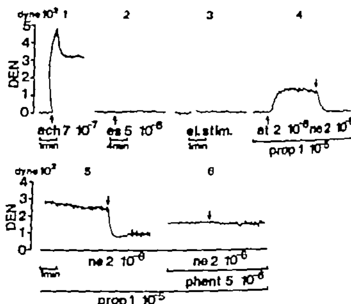


Fig. 1. Respo ses of a denervated strip from rabbit jejunum. 1 Acetylcholine 7 10^{-7} M (ach) eserine 5 10^{-6} M (es). 3 Electrical field stimulation by 1 ms rectangular pulses during 10 s at 10 Hz at 25 Hz (O) and at 50 Hz (✳). 4 Angiotensin 2 10^{-6} M (at) and norepinephrine 2 10^{-6} M (ne) in presence of propranolol 1 10^{-5} M (prop) 5 Tension of the preparation increased by carbachol (7 M) added 10 min previously At arrow norepinephrine 2 10^{-6} M (ne) in the presence of propr 1 10^{-5} M 6. Tension f the preparation increased by carbachol added 10 min previously Norepine 10⁻⁶ M (ne) in the presence of propranolol 1 10^{-5} M and phentolamine 5 10^{-6} M (phent).

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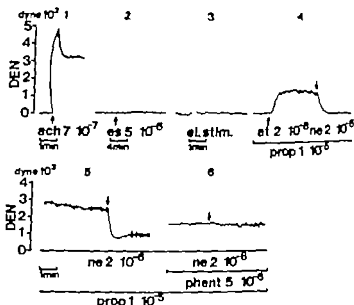


Fig. 12. Responses of a denervated strip from rabbit jejunum. 1 2 Acetylcholine 7 10^{-7} M ... eserine 5 10^{-6} M (es). 3 Electrical field stimulation by 1 ms rectangular pulses during 10 s at ... at 25 Hz (O) and at 50 Hz (✱). 4 Angiotensin 10^{-8} M (at) and norepinephrine 10^{-6} M ... presence of propranolol 1 10^{-5} M (prop). 5 Tension of the preparation increased by carbachol ... M) added 10 min previously At arrow norepinephrine 10^{-6} M (ne) in the presence of p... 1×10^{-5} M 6. Tension of the preparation increased by carbachol added 10 min previously Nore... 2 10^{-6} M (ne) in the presence of propranolol 1 10^{-5} M and phentolamine 5 10^{-6} M (phent).

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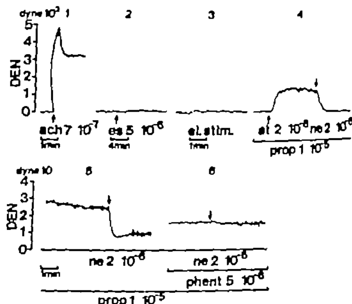


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...innervated strip from the rabbit was relaxed by -receptor stimulation. This relaxation ...d have been mediated through adrenergic α-receptors located in the smooth muscle ...and/or through α-receptors located in the neural structures of the preparation. It was ... by Vizi and Knoll (1971) that norepinephrine inhibited the release of acetylcholine ... the rabbit jejunum under resting conditions or during electrical stimulation at low ...uency (0.5 Hz). It is probable that such an inhibitory effect on cholinergic neurons could ...ribute to the action of the adrenergic drugs on the innervated rabbit jejunum. If this is ..., the response to α-receptor stimulation would be greater on contraction of the prepara... by electrical stimulation of cholinergic neurons than on contraction by exogenous acetyl...line, which mainly acts directly on smooth muscle. It was found that both norepinephrine ... phenylephrine, in the presence of propranolol, were more effective in inhibiting electrical ...tractions at a low stimulation frequency (1 Hz) than they were in inhibiting contractions ...ted by exogenous acetylcholine. Similarly phenylephrine had a stronger relaxing effect ... preparations contracted by eserine than on those contracted by exogenous acetylcholine ...s probable that contractions elicited by acetylcholine in the innervated preparation were ...some extent mediated through the stimulation of neurons. In one experiment the ganglio... blocker pentolinium was used to inhibit this action of acetylcholine on neurons. Under ...se experimental conditions phenylephrine was markedly less potent as a relaxing agent ...en the preparation was contracted by acetylcholine in the presence of pentolinium than ...en it was contracted by eserine. From these experiments it can be concluded that the ...receptor mediated relaxation in innervated rabbit jejunum is at least partially brought ...out through inhibition of the intrinsic cholinergic neurons of the preparation.

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pine The response to norepinephrine was not affected by β-adrenoceptor blockade inhibited by tolazoline, indicating an involvement of α-adrenoceptors. When the in strips had been contracted by acetylcholine a relaxing effect was exerted by norep and sometimes by phenylephrine It is probable that the contraction induced by acet in the innervated strips was, to some extent, mediated through the stimulation of α

Strips of denervated longitudinal ileum from the guinea pig contracted by acet or other drugs were not relaxed by norepinephrine in the same concentration tha the innervated strips. Nor where the preparations relaxed by phenylephrine in co tions of up to 5×10^{-4} M after blockade of β-adrenoceptors. Norepinephrine in hi centrations (3 10^{-4} M) relaxed the preparations, however This effect was not med a stimulation of α-adrenoceptors, since it was resistant to α-adrenoceptor blocking nor was it mediated by β-adrenoceptors, since neither β-receptor blocking agent combination of α and β-blockers abolished the response. Involvement of prosta in the production of this effect of norepinephrine was also improbable, since inhib prostaglandin synthesis by indomethacin did not affect the response Since a relaxing was produced by 1-(3 4-dihydroxyphenyl)-ethanol in the same molar concentratio effect was probably not mediated by adrenoceptors but by an unspecific mechan pendent on the nucleus of the catecholamine

These results indicate the absence of inhibitory α-receptors in the smooth muscle the denervated guinea pig ileum The inhibitory response observed in innervated p tions following α-receptor stimulation must therefore be due to an action on neurons neurons are probably of a cholinergic nature

Adrenergic α receptors, excitatory or inhibitory from different species generally great homogeneity with respect to the affinity constants and the activities of α-adrenc active drugs (Furchgott 1972). A difference has recently been observed between inh α-drenoceptors located in the cholinergic neurons of Auerbach s plexus of guinea pig and the guinea-pig ileocaecal sphincter and the excitatory α-adrenoceptors located smooth muscle cells of the guinea pig ileocecal sphincter and rabbit aorta (Wikberg Wikberg *et al* 1975) The presence of a pharmacologically different α-adrenoceptor in pig ileum explains a variety of hitherto conflicting data. Phenylephrine is tradition garded as a specific α-adrenergic agonist This is the case when the drug is tested on jejunum or rabbit aorta The drug had virtually no or very little effect, however when on electrically contracted guinea pig ileum or guinea pig ileocaecal sphincter (Wikberg Wikberg *et al* 1975 Wikberg 1977) Paton and Vizi (1969) reported that phenylephrin comparatively inactive in reducing the release of acetylcholine following electrical sti tion in guinea pig ileum. Phenylephrine like other 3-substituted hydroxy-phenyl- amines, was almost completely ineffective in inhibiting the peristaltic reflex in guine ileum (McDoughal and West 1954). The weak and transient effects produced by phenyle on the innervated preparations of the guinea pig in this study should be considered i light of the above discussion.

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References

Åberg, G and O Eränkö, Localization of noradrenaline and acetylcholine esterase in the taenia of the guinea pig caecum. *A ta physiol scand* 1967 69, 383–384.

Ahlquist R P and B. Levy Adrenergic receptive mechanism of the canine ileum. *J Pharmacol* 127 146–149

Ambache, N Separation of the longitudinal muscle of the rabbit's ileum as a broad sheet. *J* (Lond). 1954 *125* 53–55

Ambache, N and M A. Freeman, Atropine-resistant longitudinal muscle spasm due to excitation of non-cholinergic neurons in Auerbach s plexus. *J Physiol* (Lond) 1968 *199* 705–727

Ambache, N and A W Lessin, Classification of intestinomotor drugs by means of type D ... *J Physiol* (Lond.) 1955 *127* 449–478

Andersson, R R le of cyclic AMP and Ca++ in the metabolic and relaxing effects of catecholamines in intestinal smooth muscle *Act physiol scand* 1972. *85* 312 322.

Andersson, R. nd E. Mohme Lundholm, Studies on the relaxing action mediated by stimulation of adrenergic α nd β-receptors in taenia coli of the rabbit and guinea pig. *Acta physiol. scand* 372 384

Andersson, R. and E. Mohme Lundholm, Metabolic actions in intestinal smooth muscle associated with relaxation mediated by adrenergic α- and β-receptors. *Acta physiol. scand.* 1970. 79 244–261

Bowman W C. and M T Hall, Inhibition of rabbit i testine mediated by α- and β-adrenoceptors *J Pharmacol* 1970. *38* 399–415

Brody T M and J Di mond, Blockade of the biochemical correlates of contraction and relaxation in uterine and intestinal smooth muscle. *Ann. N Y Acad Sci* 1967 *139* 772 780.

Bucknell, A and B. Whitney A preliminary investigation of the pharmacology of the human isolated taenia coli preparation *Brit J Pharmacol* 1964 *23* 164 175.

Bülbring, E., Action of catecholamines on the smooth muscle cell membrane. I *Drug recept* H. P Rang, London: (MacMilla) 1973 1 13.

Bülbring, E. and T Tomita, Suppression of spontaneous spik generation by catecholamines in smooth muscle I guinea pig taenia coli *Proc ro Soc B* 1969 *172* 103–119

Costa, M and G Gabella, Adrenergic i nervation of the limentary tract. *Z. Zellforsch* 197 357 377

Furchgott R. F Receptors for sympathomimetic amines. I *Ciba Foundation Symposium on Adrenergic Mechanisms* ed J R. Vane London Churchill. 1960. 246–252.

Furchgott R. F., The classification of adrenoceptors (adrenergic receptors). An ev luation from the tandpoint of receptor theory I *Catecholamines. Handbook of xperimental pharmacology* Vol. X eds. H Blaschko nd E. Muschol. Berlin–Heidelberg New Y rk Springer 1972. 283–335.

Furness, J B., The origin and distribution of drenergic nerves i guinea pig colon. *Histochemie* 1969 245–306.

Furness, J B. nd M. Costa, The adrenergic innerv tion of the gastrointestinal tract. *Rev Physiol* 69 1 51

Gabella, G Fine structure of the myenteric plexus in the guinea pig ileum. *J Anat* 1972 a. *111*

Gabella, G Innervation of the intestinal muscula coat. *J Neurocytol* 1972 b. *1* 341 362.

Gershon, M D Inhibition of gastrointestinal movement by sympathetic nerve stimulation the action *J Physiol.* (Lond.) 1967 *189* 317 327

Jacobowitz, D Histochemical studies on the utonomic innervation of the gut. *J Pharmacol. ex* 1965 *149* 358–364.

Jen nson D H nd I K. M Morton The role of α- nd β-adrenergic receptors in some actions of catecholamines on intestinal smooth muscle. *J Physiol* (Lond.) 1967 *188* 387–402.

Koste litz, H W nd A. J W tt Adrenergic receptors i the guinea pig ileum *J Physiol* (Lond) *177* 11 12*P*

...UTZ, H. W., J. R. LYDON and A. J. W...TT The effects of adrenaline and isoprenaline, on inhibitory ...d β-adrenoceptors in the longitudinal muscle of the guinea pig ileum. *Brit. J. Pharmacol.* 1970, 39 ...415.

...Y Adrenergic receptors in the intestine. In *Smooth muscle* 1970, 549–557

...OLM, L. and E. MOHME-LUNDHOLM, Length of inactivated contractile elements, length tension dia...., active state and tone of vascular smooth muscle. *Acta physiol. scand.* 1966, 68, 347–359

...UGAL, M. D. and G. B. WEST The inhibition of the peristaltic reflex by sympathomimetic amines. *J. Pharmacol.* 1954 9 131 137

...NO, K. A., Adrenergic innervation of the intestinal wall studied by fluorescence microscopy *Int. J. ...ropharmacol.* 1964, 3, 379–382

...ORY A. and G. KRONBERG, The relationship between α- and β-receptor function and the liberation ...etylcholine on the coaxially stimulated guinea pig ileum. *Abstr. 4th Int. Congr. Basel*, 1969 p. 144–

...A, Y. Y. MORI and Y. W...NABE, Inhibition of the release of acetylcholine from isolated guinea pig ...m by crystalline tetrodotoxin *J. Pharmacol. exp. Ther.* 1966, 147 456–462.

...N, R. B. and D. R. WAUD Pharmacological estimation of drug receptor dissociation constants. ...stical evaluation 1 Agonists *J. Pharmacol. exp. Ther.* 1971 177 1 12.

...N, W. D. M. and J. R. V...NE, Analysis of the response of the isolated stomach to electrical stimulation ... to drugs. *J. Physiol.* (Lond.) 1963 165, 10–46

...N, W. D. M. and A. ZAR, The origin of acetylcholine released from guinea pig intestine and longitudi... muscle strips *J. Physiol.* (Lond.) 1968 194 13–33

...N, W. D. M. and E. S. VIZI, The inhibitory action of noradrenaline and adrenaline on acetylcholine ...put by guinea pig ileum longitudinal muscle strips *Brit. J. Pharmacol.* 1969 35 10–28.

...L, H. P. *Stimulant actions of volatile anaesthetics on smooth muscle. Brit. J. Pharmacol.* 1964, 22 ...–363

...ARDSON, K. C. Electron microscopic observations on Auerbach plexus in the rabbit, ...ith special ...erence to the problem of smooth muscle innervation. *Amer. J. Anat.* 1958, 103 99–136.

...ARDSON, K. C. Studies on the structure of autonomic nerves in the small intestine, correlating the ...ver impregnated image in light microscopy with the permanganate-fixed ultrastructure in electron ...croscopy *J. Anat.* 1960 94 457–472.

..., T. I. TAKA...AGI and K. TAKAGI, Pharmacological properties of electrical activities obtained from ...rons in Auerbach plexus *Jap. J. Pharmacol.* 1973 23 665–671

...UMAN, W. Zusammenhänge zwischen der Wirkung der Analgetica und Sympatomimetica auf der ...erschweinchen-Dünndarm *Naunyn-Schmiedeberg Arch. exp. Path. Pharmak.* 1958, 233 112 124

...A, D. G. G. ROSS and L. W. OSBORNE, Adrenergic innervation of the ileum of the cat. *Amer. J. Physiol.* ...71 220 347 352

..., E. S. and J. KNOLL, The effect of sympathetic nerve stimulation and guanethidine on parasympathetic ...euroeffector transmission, the inhibition of acetylcholine release. *J. Pharm. Pharmacol.* 1971 23 918–...23

...ERG, J. *Difference between α-receptors in cholinergic neurons and smooth muscle cells.* Abstract Sixth ...ternational Congress of Pharmacol. Helsinki, 1975, p. 621

...ERG, J. Differentiation of adrenergic ...-receptors in guinea pig intestine and rabbit aorta. T... be ...ublished

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References

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ANDERSSON, R. Role of cyclic AMP nd Ca^{++} in the metabolic and relaxing effects of catecholam intestinal smooth muscle. *Acta physiol. scand* 1972. *85* 312 322.

ANDERSSON, R. and E. MOHME LUNDHOLM Studies on the relaxing action mediated by stimulat adrenergic α and β-receptors in taenia coli of the rabbit and guinea pig. *Acta physiol. scand* 19 372 384

ANDERSSON, R. and E. MOHME LUNDHOLM, Metabolic actions in intestinal smooth muscle associat relaxation mediated by adrenergic α- and β-receptors. *Acta physiol scand.* 1970. *79* 44–61

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BÜLBRING, E. nd T TOMITA, Suppression of pontaneous spik generation by catecholamines smooth muscle of guinea pig taenia coli. *Proc roy Soc B* 1969 *172.* 103–119

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GABELLA, G Innervation of the intestinal muscula coat. *J Neurocytol.* 1972 b. *1* 341 36

GERSHON, M D Inhibitio of gastrointestinal movement by sympathetic nerve stimulation the action. *J Physiol* (Lond) 1967 *189* 317 327

JACOBOWITZ, D Histochemical studies on the a tonomic innervation of the gut. *J Pharmacol.* xp 1965. *149* 358 364

JENKINSON, D H. nd I K. M. MORTON, The role of α- and β-adrenergic receptors in some actio catecholamines on intestinal smooth muscle *J Ph siol.* (Lond.) 1967 *183* 387–402.

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and GHR) the perhaps dominating genetic predisposition to hypertension seems to [illegible] central neurohormonal nature, where an increased sympathetic activity and/or reacti[illegible] extrinsic stimuli, act in such a way as to cause an age-dependent, progressive blood [illegible]re elevation.

[illegible], in SHR there is good evidence for a hyper-reactivity in the central neural structures, [illegible]ding cardiovascular responses to environmental stimuli (Hallbäck and Folkow 1974 [illegible]äck 1975). Such, perhaps, fairly episodic but exaggerated cardiovascular responses in [illegible] imply an elevated mean arterial pressure, as calculated over days and weeks, which [illegible] initiates adaptive structural changes in the high pressure sections of the cardiovascular [illegible]m. In large arteries and the precapillary resistance vessels an increased wall to lumen [illegible] rapidly occurs which seems to be the prerequisite for a truly hypertensive state (*e.g.* [illegible]ow *et al.* 1973, Folkow 1975). In fact, whichever the initiating functional disturbance, [illegible]ctural cardiovascular adaptations, including left ventricular hypertrophy are gradually [illegible]loped in all types of hypertension (*cf.* Pickering 1968). These structural vascular changes [illegible] to reset both the range of resistance vessel control and the baroreceptor function to a [illegible]er pressure equilibrium. It further implies a vascular hyper-reactivity which potentiates [illegible]tatory functional influences and hence creates a positive feedback interaction with [illegible]h influences.

[illegible]uch attention has been focussed on the possibility of a primary *renal* involvement, [illegible]east in some variants of essential hypertension in man or genetic hypertension in animals. [illegible]wever regardless of the aetiology of hypertension the kidney will sooner or later become [illegible]olved, both because there are many ways in which its function can be reset and because [illegible] vascular bed seems to be particularly vulnerable, and hence disturbed, by increases in [illegible]e pressure load. Although there seems to be no primary renal factor contributing to the [illegible]tation of hypertension in GHR and SHR, other strains of "spontaneously" hypertensive [illegible]ts have been developed in which renal factors seem to play a primary and dominant role. [illegible]hus Dahl, Heine and Tassinari (1962) have developed a strain of rats, "the Brookhaven [illegible]rain of hypertension-sensitive rats. These rats develop hypertension when exposed to [illegible]creases in salt load and the salt "sensitivity" can be transferred to salt-resistant rats by [illegible]dney transplantation (Dahl, Heine and Thompson 1974).

Renal factors, but evidently of somewhat different nature, also contribute to the development of hypertension in the Milan strain of hypertensive rats (MHS) (Bianchi, Fox and Imbasciati 1973). Thus, MHS does not need a high salt diet to develop hypertension but compared with normotensive controls the renal filtration capacity seems to be reduced in the prehypertensive stage in relation to the tubular reabsorptive capacity. MHS exhibits signs of an increased plasma volume in early age which seems related to modest salt retention, but when blood pressure increases these changes revert back towards normal (Bianchi *et al.* 1975 a, b). Thus, it seems that the kidneys in the MHS need a higher perfusion pressure to operate in normal way. Also, if the kidneys are crosstransplanted between MHS and their appropriate normotensive controls, the hypertensive stage goes with the kidneys from the hypertensive animals (Bianchi *et al.* 1974).

The present study was undertaken to investigate some aspects of the hemodynamic situation in this MHS type of "volume hypertension". At the same time the neurogenic cardio-

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From the Department of Physiology University of Göteborg, S eden

Cardiovascular Control in the Milan Strain of Spontaneously Hypertensive Rat (MHS) at "Rest" and during Acute Mental "Stress"

By

MARGARETA HALLBÄCK, JOHN V JONES GIUSEPPE BIANCHI and BJÖRN FOLKOW

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Abstract

HALLBÄCK, M., J V JONES, G BIANCHI and B FOLKOW *Cardiovascular control in the Milan strain of spontaneously hypertensive rat (MHS) at "rest" and during acute mental "stress"* Acta physiol. scand. 1977 99 208–216.

The cardiovascula responses to acute mental "stress were compared in the Milan strain of spontaneously hypertensive rats (MHS) and in normotensive control rats (NR). Blood pressure and heart rate were followed in pairs of awake MHS nd NR, while defence reactions were provoked by alerting st (noise, vibration). N differences were noted between the two groups in response t stress although resting heart rate i MHS was lower tha in NR. Administration of tropine or propranolol to MHS and NR showed the MHS to have higher resting vagal tone and lower sympathetic tone than the NR. Subsequent (at least two weeks later) hemodynamic invest gation, under nembutal nesthesia, showed no difference in cardiac output between MHS and NR but a higher stroke volume, presumably related to the lower heart rate i MHS Thus, total peripheral resistance was increased i MHS as was the ratio left ventricular weight/body weight, and i good proportion to the blood pressure rise Thus MHS differ substantially both their responses t "stress nd also hemodynamically from the Okamoto strain of spontaneously hypertensive rat (SHR), being the so far most studied and best known model of essential hypertension in man In MHS the hypertension is more of a ystolic type and is of primarily renal origin As such, MHS provide another model f investigating the polygenic nat re of hypertension in man.

The search for an adequate animal model of essential hypertension in man has led to the development of several different strains of genetically or "spontaneously hypertensive" rats. The first to be developed was the New Zealand strain of genetically hypertensive rat (GHR), based on selective breeding of Wistar rats with above normal blood pressure levels (Smirk and Hall 1958). However the most commonly used and hence most extensively studied type of genetically hypertensive rat is the spontaneously hypertensive rat (SHR) developed from the Kyoto Wistar strain by Okamoto and Aoki (1963) In both these stra

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...LE 1 The resting heart rate (HR) and mean arterial blood pressure (MBP) are given for both the Milan strain of spontaneously hypertensive rat (MHS) and their normotensive controls (NR). The change from resting values in one group given propranolol and another given atropine are shown. All values are mean ± S.E.

	Resting		Change from resting value			
			After propranolol		After atropine	
	MBP	HR	MBP	HR	MBP	HR
HS	118 ± 2	309 ± 6	+ 18 ± 2	51 ± 8	+ 1 ± 2	+ 68 ± 6
R	107 ± 2	351 ± 8	+ 12 ± 1	74 ± 10	− 2 ± 1	+ 43 ± 15
	= 16	= 16	= 8	= 8	= 8	= 8
	0.01	0.001	0.05	0.05		0.01

...olated room, and when the rats had recovered from the superficial ether anesthesia ...ee Table I). In this situation of paired comparison in identical environments a mild hypertension was evident in the MHS, the mean pressure being 118 ± 2 mmHg as compared with 107 ± 2 mmHg in NR ($p < 0.01$), i.e. 10% difference. Basal heart rate in MHS was ...en, however, reduced by some 10 per cent as compared to NR, being 309 ± 6 beats/min and 351 ± 8 beats/min, respectively ($p < 0.001$).

When exposed to brief periods of stressful stimuli MHS and NR exhibited largely similar responses, i.e. their increases in heart rate and blood pressure *did not substantially differ* either in direction or extent, as shown in the lower part of Fig. 1. The mean increase in heart rate during the *30 s stimulatory* period showed a tendency to be slightly more pronounced in MHS, being here 13 ± 5 beats/min compared with 6 ± 4 in NR but the difference was not statistically significant. The mean increase in blood pressure during the stress period was only 2 ± 2 mmHg in MHS and 3 ± 1 mmHg in NR. These responses are thus quite modest in extent compared with the powerful ones induced in the Okamoto strain of SHR compared with their controls, when exposed to the same stimuli (*see Fig. 1 upper part*).

Neurogenic control. To evaluate possible differences between MHS and NR concerning neurogenic control of the heart, the *16 pairs of rats* were divided into two groups. One group (containing 8 MHS and 8 NR) received propranolol after having been tested in the stress box. The *degree of adrenergic influence* on the heart in the awake "resting" MHS and NR was judged by the individual change in heart rate after administration of propranolol. These changes were significantly smaller in MHS than in NR in this particular situation of paired comparison during "rest" (see Table I). The remaining rats were given atropine. A greater increase in heart rate was then evident in MHS than in NR which thus suggested a more pronounced vagal tonic influence on the MHS heart (Table I).

Thus, the heart rate of the MHS was relatively less affected by propranolol than by atropine, whereas the reverse was true for the NR. This indicates that in the Milan strain of spontaneously hypertensive rats the heart is more influenced by vagal control than is the case in their appropriate NR, at least in the prevailing situation which was identical for both. Furthermore the sympathetic cardiac activity seems less pronounced in MHS than that present in NR. The fact that the resting heart rate in MHS in this type of paired

vascular control in MHS was explored during both basal conditions and during episodes of stressful stimulation in paired comparisons with their appropriate normotensive controls.

Methods

Sixteen rats of the Milan hypertensive strain and 16 normotensive Milan Wistar rats (NR) were used in "stress" expts. For hemodynamic analysis 10 rats out of each group were used, having been earlier used for brief exposures to environmental stimuli to explore their cardiovascular reactivity. At least weeks elapsed between these two types of acute expts. All rats used were males aged 4–5 months, weighing between 300 and 350 g. The "stress" expts. were always carried out on pairs of one MHS and one NR. Their tail arteries were cannulated during brief ether anesthesia, the incision sutured and local anesthetic applied around the incision. The rats were placed in the stress box in which they could be exposed to 30 s periods of brief stress stimulations in the form of loud highfrequency noise and/or vibrations of the box (for further details see Hallbäck and Folkow 1974). The rats were allowed to recover from the anesthesia for one hour before the experiment started. Blood pressure and heart rate were continuously recorded from the tail artery cannula which was connected to a Statham pressure transducer operating on a Grass Polygraph. Presented mean values during basal conditions are averages of at least 3 recordings in each rat during a 40 min period prior to any stress stimulation. The changes in heart rate and blood pressure during the 30 s stress stimuli and the 30 poststimulatory s are presented as follows. The changes from prestimulatory values are calculated for each third second in each rat. After which the difference in response between the paired MHS and NR at each third second interval is calculated. The mean of these paired differences, and the standard error of the mean differences, are then calculated as well as the significance of differences in response (see further Hallbäck and Folkow 1974). A significant difference in total response is considered to be present when 6 out of the 10 observations during stress stimuli, or 12 out of the 20 observations, differ above 95 per cent probability.

In the course of the expts. atropine, 1 mg/kg, was given to block the vagal cardiac effects and propranolol, 3 mg/kg, was given to block the sympathetic cardiac effects. The drugs were administered to the awake animals via the tail artery in no larger volumes than 0.5 ml. The respective drug effects were calculated in terms of individual changes in heart rate and blood pressure in each rat from the pre-drug control values. The presented mean values and standard errors of the mean, as well as level of significance, were calculated by means of paired t test.

The rats used in the hemodynamic study were first briefly anesthetized with ether. Their right femoral artery and vein were then cannulated, after which the rats were slowly given 50 mg/kg b.w. of urethane as an i.v. infusion. The tail artery was cannulated for blood pressure and heart rate measurements. The cannula was connected to a Statham pressure transducer writing on a Grass Polygraph model 7. Heparin 500 IU/kg b.w. was given to prevent clotting of blood. The dye dilution technique was used for cardiac output measurements. By means of a step dispenser syringe 0.04 ml of a cardiogreen solution (2 mg/ml) was rapidly injected into the femoral vein. For each recording of the arterial concentration of dye no more than 0.35 ml of blood (less than 5 per cent of the total blood volume) was sucked from the femoral artery into a specially designed low volume (0.05 ml) densitometer cuvett (for reference see Albrecht et al. 19[illegible]). After each determination the blood was rapidly reinfused. The densitometer was connected to a Servogor for recording of the dye dilution curves. For calculating the dye dilution recordings, a digitizer was connected to a Hewlett Packard calculator was used. Statistical evaluation of the hemodynamic study was carried out by means of the group comparison t-test.

Results

Stress experiments. The cardiovascular responses to brief episodes of stressful stimuli (sudden loud noise or vibrations of the box) were tested in 16 pairs of MHS and their normotensive Milan control rats.

"Basal" values of blood pressure and heart rate prior to the stress episodes were obtained in the awake rats after 1 h accomodation to the stress-[illegible] in a semidark sound-

ean pressure was 97±4 mm Hg. Also during nembutal anaesthesia and surgically l for the cardiac output measurements, the heart rate of the MHS was significantly an that in NR, being 320±9 beats/min vs. 383±9 beats/min ($p < 0.001$). There ever no difference between MHS and NR concerning cardiac output, which was 3 ml/min 100 g in MHS and 21 7±1.3 ml/min 100 g in NR. Because of the lower te, stroke volume was 16 per cent greater in MHS than in NR ($p < 0.05$) being 66±4 vs. 57±3 µl/100 g.

lows from the mean arterial pressure and cardiac output figures that total peri- resistance was elevated by about 15 per cent in MHS. Left ventricular weight ly weight was some 13 per cent higher in MHS than in NR as a reflection of the d afterload for the left ventricle (see Table II). It thus appears that the increase in volume, in total peripheral resistance and degree of hypertrophic adaptation of the uscle are in good proportion to the increase in mean arterial pressure in these rats rly mild chronic elevation of mean arterial pressure.

Discussion

present experimental situation the blood pressure elevation was found to be fairly n MHS, as judged by only some 15 per cent increase in mean arterial blood pressure compared to control rats (NR). The technique used in the present study of direct rterial blood pressure recording from the tail artery is the standard method employed department. However when mean arterial pressure is instead measured *via* the carotid the difference between MHS and NR is in the order of some 25 per cent (Bianchi, publ.) It thus appears as if MHS also display an increased resistance along more mal arteries as well as along the true resistance vessels, since the difference in pressure en the carotid and tail arteries is considerably smaller in NR than in MHS.

wever irrespective of such divergences in mean pressure level along the arterial tree, MHS hypertension seems to be more mild than in SHR and to involve some genetically rmined renal abnormality (Bianchi, Fox and Imbasciati 1973 Bianchi *et al.* 1975 a, b). hemodynamic consequences of such renal abnormality which tends to cause an ased plasma volume, have been thoroughly discussed by *e.g.* Ledingham and Cohen 4), Guyton and Coleman (1969), Bianchi, Tenconi and Lucca (1970) and Ledingham 1) It has been suggested, that a primary overfilling of the circulatory system leads to ncreased cardiac filling pressure, and hence to an increased stroke volume and cardiac put. In response to the increased output "whole body auto-regulation is assumed take place, upon which cardiac output and blood volume return towards normal yton, Granger and Coleman 1971).

The results from the present hemodynamic investigation in MHS may in general terms, ow the above-mentioned sequence of events. Thus, the increased stroke volume found in HS, in combination with signs of a reduced sympathetic influence on the heart, suggest t a raised cardiac filling pressure was present. The increased stroke volume may explain, least in part, why systolic pressures in MHS differ particularly much from those in NR *et al.* 1975 a, b), while the differences in mean arterial pressure were more modest

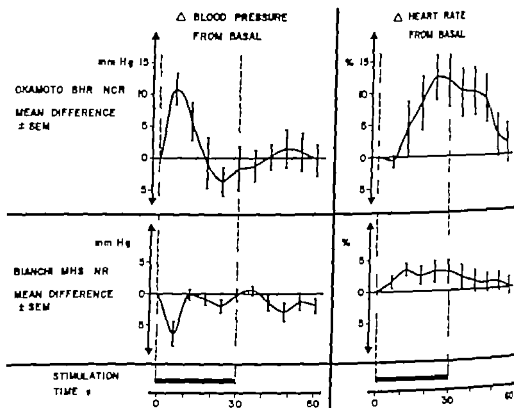


Fig. 1 Mean difference (± S.E.) in blood pressure (left) and heart rat (right) responses to stress stimuli between 9 pairs of SHR of the Okamoto strain and thei co trols, NCR (from Hallbäck 1975) and between 18 MHS rats and thei controls, NR (lower panel) Changes in heart rate are expressed in per cent of resting heart rate.

comparison is significantly lower than in NR may thus be due to such opposite, but evidently modest, shifts in vagal and sympathetic control of the heart in MHS

Hemodynamic study 10 MHS and 10 NR were investigated during nembutal anesthesia under identical experimental circumstances As shown in Table II mean arterial pressure was 110 ± 3 mmHg in MHS being thus elevated some 15 per cent compared to that of

TABLE II Mean ± S.E. values for mean arterial blood pressure (MBP), heart rat (HR), cardiac output (CO), strok volume (SV) total peripheral resistance (TPR) nd left ventricular eight as percentage of total body weight are given for both the M lan strain of spontaneously hypertensive rat (MHS) and their normotensive controls (NR). The ratios of these values in MHS/NR are given as are the statistical differences between them

	MBP	HR	CO ml/min 100 g	SV μl/100 g	TPR mmHg/ml min 100 g	Left ventric. weight/body weight,
MHS n = 10	110 ± 3	320 ± 9	21 1 ± 1.3	66 ± 4	5._ ± 0.4	0.210 ± 0 01
NR n = 10	97 ± 4	383 ± 9	21 7 ± 1.3	57 ± 3	4.5 ± 0.3	0.184 ± 0 00
MHS/NR	1 13	0.84	0.97	1 16	1 15	1 14
–p 0 05	–p 0 01	–p 0.001				

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by the considerably lower heart rate in MHS than in NR. This cardiac slowing in MH
seems primarily to be due to an increase in vagal activity and reduction sympathetic dr
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Following complete blockade of cardiac nervous control heart rate was essentially the sa
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Total peripheral resistance was increased in MHS A "whole body autoregulation
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In MHS both the degree of hypertension and the extent of structural vascular adaptatio
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ever it appears, from the present findings on mature MHS in the established phase rpertension, as if cardiac performance is then reflexly damped resulting in a lower heart hile stroke olume remains somewhat raised. Further analyses of the degree of struc vascular changes and of cardiac receptor and baroreceptor function are required to y the complex hemodynamic situation in MHS However there is no doubt that this of primary genetic hypertension in rats differs from that in SHR or in GHR in several rtant respects.

nvestigation was supported by grants from the Swedish Medical Research Council (B77 14X-00016- the Swedish National Association against Heart and Chest Diseases and the Medical Faculty rsity of Goteborg John V Jones is Brutish Medical Research Council Travelling Fellow for 1975-76 Italy generously covered part of the expenses for technician The skilful technical assistance of Ulla ... gratefully acknowledged.

References

...cki, I M Hallbäck, S. Julius, Y Lundgren, L. S aor, L. Weiss and B. Folkow Arterial sure, cardiac output and systemic resistance before and after pithing in normotensive and spontaneously hypertensive rats. *Acta physiol. scand* 1975. 94 378–385.
...i, G P G Bark, U Fox, L. Duzzi, D. Paoletti and A. M. Giovanetti, Changes in renin, ater nce and sodium balance during development of high blood pressure in genetically hypertensive rats. *Res* 1975 *36 and 37* Suppl. I. I. 153–I 161
...i, G., P G Bark, U Fox and D Paoletti, Kidney function and blood pressure in genetic type hypertension *Proc. 6th Int Congr Nephrol* Karger A G Basel. 1975 b In press
...i, G., U. Fox, G F Difrancesco, A. M. Giovanetti and D Paoletti, Blood pressure changes duced by kidney cross-transplantation between spontaneously hypertensive rats (SHR) and normotensive rats (NR). *Clin. Sci Mol Med.* 1974. 47 435–448
...i, G U. Fox and E. Imbasciati, The development of new strain of spontaneously hypertensive *Life Sci* 1973. 14 339–347
...i, G L. T Tinconi and R. Lucca, Effect in the conscious dog of constriction of the renal artery ...the remaining kidney on haemodynamics, sodium balance, body fluid volumes, plasma renin centration and pressor responsiveness to angiotensin *Clin. Sci.* 1970. 38 741 766
..., L K., M Heine and L. Tassinari, Effects of chronic excess salt ingestion Evidence that genetic ...rs play an important role in susceptibility to experimental hypertension. *J exp Med.* 1962. 115. ...-1198
..., L K M Heine and K. Thompson, Genetic influence of the kidneys on blood pressure Evidence ...chronic renal homografts in rats with opposite predispositions t hypertension. *Circulat Res.* ... 34 94–101
...w B Central neurohormonal mechanisms in spontaneously hypertensive rats compared with ...an essential hypertension *Clin. Sci Mol. Med.* 1975. 48. Suppl 2. 205–214.
..., B M Hallbäck, Y Lundgren, R. Sivertsson and L. Weiss, Importance of adaptive changes ...ascular design for establishment of primary hypertension, studied in man and in spontaneously ...tensive rats *Circulat Res* 1973. *32 and 33* Suppl. I I 2–I 13.
...w B M Hallbäck, Y Lundgren, L. Weiss, I. Albrecht and S. Julius, Analyses of design and ...ctivity" of series-coupled vascular sections in spontaneously hypertensive rats (SHR). *Acta physiol.* ... 1974 90 634–656
...n, A C. and T G. Coleman, Quantitative analysis of the pathophysiology of hypertension. *Circulat* ... 1969 24 Suppl. I. I 1–I 19.
...n, A. C., H J Granger and T G. Coleman, Autoregulation of the total systemic circulation and ...lation to control of cardiac output and arterial pressure *Circulat. Res.* 1971 *28 and 29.* Suppl. ...93–I. 97
...ck, M., Interaction between central neurogenic mechanisms and changes cardiovascular design ...mary hypertension. *Acta physiol. scand.* 1975 Suppl. 424.

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Total peripheral resistance was increased in MHS A "whole body autoregulation as described above may theoretically account for such a resistance increase, although, of course, other mechanisms may well contribute or may even be more important. It may be said that a chronic type of truly 'whole body autoregulation" is constituted by the widespread structural increase of wall/lumen ratio that occurs throughout the systemic precapillary resistance vessels in any type of hypertension (cf Folkow et al 1973) This "structural autoregulation has in various ways been demonstrated to occur rapidly as a local adaptive process in response to even a few weeks of average increase in pressure load (Folkow et al 1973 Lundgren 1974, Weiss 1974 Hallbäck 1975)

In MHS both the degree of hypertension and the extent of structural vascular adaptation, as judged by the degree of left ventricular weight increase, are decidedly less than in SHR. No analysis of the resistance vessel design has been performed in MHS so far However, a mild degree of "structural autoregulation" is likely to be present which may at least partly explain the rise in systemic resistance in this type of genetic hypertension. The reason is that the degree of left ventricular hypertrophy and the degree of structural adaptation of the resistance vessel tend in general to run closely parallel when the average blood pressure becomes elevated (Lundgren 1974) In MHS left ventricular hypertrophy was evident as a 15 per cent increase over NR as judged from the ratio left ventricular weight/body weight.

The present findings thus show that the stroke volume is somewhat increased in MHS, by which these rats differ substantially from other types of spontaneous hypertension in rats, such as the SHR. Thus, in contrast to MHS when SHR are in their established phase of hypertension, their stroke volume is rather lower than in their controls (Pfeffer and Frohlich 1973) Furthermore, in MHS hypertension, which primarily seems to involve some genetically linked alteration in renal function (Bianchi et al 1975 a, b), the present study has shown that there is no sign of any central neurohormonal hyper reactivity" of the type met with in SHR (Hallbäck and Folkow 1974) Thus, unlike SHR, MHS did not exhibit any more pronounced cardiovascular responses to graded stress stimuli as compared with their normotensive controls. Furthermore, they did not show any evidence for an enhanced neurogenic "drive" to the cardiovascular system during "rest" If anything, neurogenic cardiovascular influences in MHS rather appear to act in a manner such as to "damp the hypertensive state, as judged from the effects of selective nervous blockade of the heart.

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Conjugated Catechol Derivatives in a Transplantable Islet Cell Tumour of the Golden Hamster

By

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Abstract

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...o glucuronidated catechol derivatives have been identified in a transplantable islet cell tumour of the ...den hamster, dopamine-4-O-glucuronide and 3-methoxytyramine-4-O-glucuronide. L-dopa is rapidly ...abolized in the tumour to one or both of these glucuronides. Incubation of tumour homogenates in the ...ence of β-glucuronidase shows that dopamine-4-O-glucuronide is present in the tumour in extremely ...h concentrations.

...transplantable, insulin-producing islet cell tumour originally described by Kirkman (1962) has been found to contain dopa, dopamine and 5-hydroxytryptamine (Cegrell *et al.* 1969 a) as well as enzymes involved in the synthesis and break-down of monoamines (Cegrell *et al.* 1969 b, Axelsson *et al.* 1970). Cegrell *et al.* (1969 a) have also noted the presence of a substance which is fluorogenic in the Falck–Hillarp formaldehyde method (Falck 1962, Falck *et al.* 1962), like the monoamines and their immediate amino acid precursors, but which could not be visualized on paper chromatograms with $K_4Fe(CN)_6$. In this study two substances corresponding to this earlier observation were isolated from the tumour and identified.

Material and Methods

Islet cell tumour tissue was transplanted subcutaneously to adult golden hamsters and allowed to grow for 4–8 weeks. The tumours then weighed 4–10 g and showed no macroscopic signs of necrosis.

In some expts. tumour tissue was homogenized in 0.4 M ice-cold perchloric acid, and the supernatant obtained on centrifugation was purified on a Dowex 50 W-X4, 200–400 mesh, cation exchange column (3 × 54 mm), equilibrated with 1 M sodium acetate buffer pH 6.0. The elution was performed with 16 ml 1 M HCl. The eluate was collected in two portions of 8 ml each. The unknown substances were estimated by transforming them to fluorophores with formaldehyde and hydrogen peroxide at pH 1 according to the tryptamine method of Hess and Udenfriend (1959). For paper chromatography the eluates were evaporated

HALLBÄCK, M. Consequence of social isolation on blood pressure, cardiovascular reactivity and design in spontaneously hypertensive rats. *Acta physiol. scand.* 1975. *93*. 455–465.

HALLBÄCK, M. and B. FOLKOW. Cardiovascular responses to acute mental "stress" in spontaneously hypertensive rats. *Acta physiol. scand.* 1974. *90*. 684–698.

LEDINGHAM, J. M. Mechanisms in renal hypertension. *Proc. roy. Soc. Med.* 1971. *64*. 409–418.

LEDINGHAM, J. M. and R. D. COHEN. Changes in extracellular fluid volume and cardiac output during the development of experimental renal hypertension. *Canad. med. Ass. J.* 1964. *90*. 292–294.

LUNDGREN, Y. Adaptive changes in cardiovascular design in spontaneous and renal hypertension. *Acta physiol. scand.* 1974. Suppl. 408.

OKAMOTO, K. and K. AOKI. Development of a strain of spontaneously hypertensive rats. *Jap. Circulat. J.* 1963. *27*. 282–293.

PFEFFER, M. A. and E. D. FROHLICH. Hemodynamic and myocardial function in young and old normotensive and spontaneously hypertensive rats. *Circulat. Res.* 1973. *32 and 33*. Suppl. I. I 28–I 35.

PICKERING, G. High blood pressure. London, J. & A. Churchill Ltd. 1968.

SMIRK, F. H. and W. H. HALL. Inherited hypertension in rats. *Nature* (Lond.) 1958. *182*. 727–728.

WEISS, L. Aspects of the relation between functional and structural cardiovascular factors in primary hypertension. *Acta physiol. scand.* 1974. Suppl. 409.

ours once dopa injections increased, and dopa decarboxylase inhibition decreased, the rescence intensities produced by these 2 substances. Thus, it seemed reasonable to investigate the hypothesis that the 2 compounds were substituted catechol derivatives.

6 tumours ere homogenized separately in 6 M HCl (20 ml) and centrifuged for 10 min 17 000 rpm. The supernatants were either refluxed for 3 h (5 tumours) or stored at 4 C 3 h. The acid was evaporated and the residue was dissolved in 0.4 M perchloric acid) ml) and adsorbed onto Al_2O_3, a dopamine determination was made according to Anton & Sayre (1964). After refluxing, the dopamine content was 10 ± 1.8 µg/g (mean $\pm$ S.D.) tumour tissue; in the control stored at +4°C the content was significantly less, 3.4 ± 0.56 µg/g (p 0.001).

In another series of expts., 3 tumours were homogenized in 0.4 M perchloric acid and the supernatant was passed through the cation resin. The first portions (8 ml) of the eluates were pooled and evaporated to near dryness and the residue was extracted with acetone and centrifuged. The supernatant was again evaporated to a small volume and divided into 3 equal parts which were applied to a Whatman No. 1 paper. After developing in the same system as above, the paper was cut longitudinally into 3 strips. One was treated with formaldehyde gas to localize the two substances with Rf values 0.10 and 0.28. The corresponding areas as well as the areas above and below each spot, i.e. in all 6 areas from each of the remaining strips were extracted with 0.1 M HCl. The 6 extracts from one strip were kept at +4°C for 3 h, and the remaining 6 extracts were refluxed for 3 h in 6 M HCl. In determinations according to Anton and Sayre dopamine could be detected only in the refluxed extract from the spot with Rf value 0.10.

In a third series of expts. focused on the substance with Rf value 0.28, the same procedure was followed up to and including the hydrolysis. The 6 extracts were prepared for gas chromatography-mass spectrometry (GC-MS) by evaporation to dryness, and the formation of derivatives with 40 µl trifluoroacetic acid anhydride in 20 µl ethyl acetate at room temperature for 1/2 h. The solution was then evaporated to dryness and the residue was taken up in 1 trifluoroacetic acid anhydride in ethyl acetate. Mass spectra were run on an LKB 2091 instrument fitted with a 3% OV 17 column (helium flow rate 20 ml/min column temperature 130°C ion source 270°C ionizing electron energy 70 eV).

Only the derivative from the refluxed extract of the spot gave a significant gas chromatographic peak. The mass spectrum recorded from this peak matched the spectrum recorded from the trifluoroacetoxy derivative of 4-hydroxy-3-methoxyphenylethylamine (Fig. 1). The 2 samples also had the same retention time on gas chromatography. The derivative of 3-hydroxy-4-methoxyphenylethylamine gives a mass spectrum that is significantly different, especially in the intensity of the peak at m/e 233 ($M-CH_2NHCOCF_3$). If the fragment resulting from this decomposition has the structure of a methoxy-trifluoroacetoxytropylium ion (Tait et al. 1962), it should be identical for the 2 isomers. However retention of the benzyl structure ould allow resonance stabilization with participation of the electrons on the p-methoxy group, giving a stable quinoid structure (Fig. 2). For the m-methoxy isomer the strongly electronegative trifluoroacetoxy group in the para-position would not give the same mesomeric effect. The lower stability of the 3-methoxy-4-trifluoroacetoxybenzyl cation should promote further decomposition. This probably occurs with elimination of carbon monoxide

to near dryness at room temperature and the residue was extracted with acetone. After centrifugation supernatant was evaporated to small volume, applied to paper (Whatman No 1) and run in phenol - M HCl (9 1). The determination of catechol derivatives was made by the spectrophotofluorometric method of Anton nd Sayre (1964) using an Aminco-Bowman spectrofluorometer Other methodological details will be described below

L-dopa, dopamine and Type H 2 crude β-glucuro idase solutio from Helix pomatia (containing sulfatase), were purchased from Sigma (β-glucuronidase activity: app 100 000 Fishman units/ml; sulfatase activity: 1 ml will hydrolyze up to 10 000 μmol of p-nitrocatechol sulfate per hr at pH 5 at 37°C) The dopa decarboxylase inhibitor NSD 1015 (m-hydroxybenzyl hydrazine) was obtained from Smith and Nephew

Results

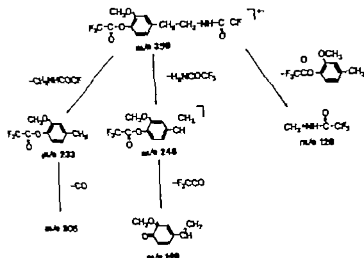
Introductory paper chromatographic and spectrophotofluorometric analyses In 5 expts eluate portions from the cation resin were chromatographed on paper with authentic L-dopa and dopamine Three chromatograms were exposed to dry formaldehyde gas at 80 for 1 h (*i.e* the same reaction conditions used in the Falck-Hillarp histochemical method for the visualization of certain phenylethyl and indolylethyl derivatives) The first 8 ml from the ion exchange column gave 2 intensely blue fluorescing spots with Rf values of 0 10 and 0.28 respectively The second eluate portion showed one spot with an intense yellow-green fluorescence and the same Rf value as authentic dopamine. Two chromatograms were sprayed with 0 44% K Fe(CN)₆ (pH 7 4) Only the dopamine spots appeared.

In a second series of 4 expts, the material from the first 8 ml of the eluate was divided into 2 equal portions and chromatographed on paper One half of the paper was treated with formaldehyde gas to localize the 2 blue fluorescent spots. The corresponding areas of the other half of the paper were extracted with 0 1 M HCl and the extracts were treated according to the tryptamine method of Hess and Udenfriend (1959) to produce fluorophores. In both cases a significant fluorescence was obtained with excitation/emission maxima at 350/455 nm (uncorrected instrumental values) These spectral characteristics are clearly different from those displayed by the tryptophan and tryptamine fluorophores which have their excitation/emission peaks at 365/440 nm.

Effect of dopa injections and dopa decarboxylase inhibition. 6 out of 12 tumour-carrying animals were injected intraperitoneally with L-dopa 100 mg/kg b w and killed 2 h after the injections. The tumours were removed and extracts prepared as above were purified and treated to produce fluorophores according to Hess and Udenfriend A comparison of the fluorescence intensities obtained from the 2 groups showed a mean increase by a factor of 2.3 ($p < 0.001$) in the tumours from the animals treated with dopa

In another series, 6 animals received NSD 1015 100 mg/kg b.w., and the tumours were removed 1 5 h after the injections. 6 tumours from untreated animals served as controls. The dopa decarboxylase inhibition produced a mean decrease in fluorescence intensity by a factor of 0 63 ($p < 0.01$).

Acid hydrolysis. The data above show that the unknown substances have the molecular requirements for condensing with formaldehyde to give intensely fluorescent derivatives in dry milieu (*cf* Björklund *et al* 1972) as well as in solution. They cannot however be visualized on paper with K Fe(CN)₆ in the same way as dopa and the biogenic catecholamines. One or both of the substances seemed to be connected wi[illegible] etabolism in the



Mass spectral fragmentation of 4-hydroxy-3-methoxyphenylethylamine trifluoroacetyl derivate

…d in 0.1 M sodium acetate buffer (1 g tissue/3 ml buffer) and incubated with Type …β-glucuronidase solution. The dopamine content was determined according to Anton …Sayre (1964). The results are summarized in Table I. Glucuronic acid was identified …ding to Karting and Wegschaider (1971). It is evident from Table I that addition of …H-2 extract to the incubation medium results in a pronounced increase of free dopa… This is presumably due to the β-glucuronidase activity since inhibition of the sulfatase …ity of the extract by phosphate ions did not decrease the amount of dopamine formed …column III and IV in Table I). Incubation without added β-glucuronidase resulted in …t formation of dopamine (*cf* column I and II in Table I, $p < 0.01$). This would indicate …β-glucuronidase activity in the tumour since the dopa decarboxylase known to be …ent (Cegrell *et al.* 1969 b), is inactivated at the pH used during incubation.

…he presence of β-glucuronidase activity in the tumour tissue was established using the

…LE I Formation of dopamine from hamster insuloma tissue expressed as µg DA/g tumour tissue (10 ml of homogenate were incubated at +37°C for 1 hr at pH 4.5 for each determination).

…	I Direct extraction with 0.4 M perchloric acid at ±0°C	II Incubation without added enzymes	III Incubation with 1 ml Type H-2 enzyme	IV Incubation with 1 ml Type H-2 enzyme and 0.1 M sodium phosphate
	8.7	16	63	
	9.8	11	40	
	11.0	17	55	48
	8.9	13	43	47
	8.5		49	45

…n β-glucuronidase and sulfatase activity see Material and Methods

Fig. 1 Mass spectra of trifluoroacetyl derivatives of 4-hydroxy-3-methoxyphenylethylamine and 3-hydroxy-4-methoxyphenylethylamine.

(Costa *et al* 1972) from fragment m/e 233 giving the peak m/e 205 which is not present in the mass spectrum of 4-methoxy-3-trifluoroacetoxy isomer (Fig. 3).

The 2 unknown substances thus yield dopamine and 3-methoxytyramine respectively upon hydrolysis in refluxing acid.

Demonstration of dopamine glucuronide by enzymatic hydrolysis 5 tumours were homo-

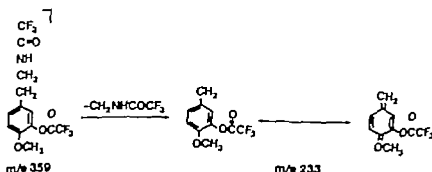


Fig. 2. Mass spectral fragmentation t a resonance stabilized 4-metho y 3-tr ybenzyl cation

Discussion

studies using the Falck Hillarp histochemical method, have shown that exposure to ehyde gas gives an intense blue fluorescence in hamster insuloma cells (Cegrell *et al.* The spectral characteristics were different from those displayed by the fluorophores techol and indole derivatives which have been studied according to this method in and model systems. This indicated the presence in the insuloma of one or several fied fluorogenic compounds whose formaldehyde fluorophores had an emission t considerably lower wave lengths than biogenic catechol and indole derivatives l so far

the present work, paper chromatographic analyses (phenol −0.1 M HCl 9 1) have strated 2 compounds in the insuloma tissue with Rf values 0 10 and 0.28 respectively velop blue fluorescence when the paper is exposed to formaldehyde gas under the e conditions used in the histochemical formaldehyde method.

l hydrolysis of the slow moving compound and of homogenized tumour tissue result formation of dopamine. The substance should thus be dopamine derivative, possibly -sulfate or the *O*-glucuronide. Incubation of tumour homogenates in the presence of uronidase and sulfatase demonstrated considerable formation of dopamine due to -glucuronidase activity The magnitude of this amine formation did not change upon tion of the sulfatase. The compound present in the tumour should thus be a dopamine-curonide.

is dopamine-glucuronide could be a mono- or a diglucuronide though from a biological of view it seems highly improbable that the diglucuronide is formed. To further eluci-this problem it was of importance to compare the properties of the substance in hamster oma with those of synthetic dopamine-glucuronide, preferable 4-*O*-(β-D-glucopyranosi-nic acid)-dopamine (DA-4-*O*-glucuronide). To our knowledge a synthesis of this com-nd has not hitherto been described. A synthetic method had therefore to be worked out mson and Rosengren 1976). Paper chromatograms showed that the slow-moving sub-ce from tumour extracts had the same Rf value as the synthetic substance and moreover the formaldehyde-induced fluorophores of both substances had the same fluorescence racteristics Convincing evidence that the dopamine-glucuronide isolated from insuloma ue is not diglucuronide but a monoglucuronide substituted in the 4-position was ained by eluting both this and the synthetic compound from paper chromatograms and ucing fluorophore formation with formaldehyde and hydrogen peroxide in solution ac ding to Hess and Udenfriend (1959). The spectral characteristics of the two fluorophores orded at different pH values revealed a wave-length shift of the excitation maximum and pH dependent fluorescence intensity These phenomena are significant for fluorophores ving a quinoidal structure implying that they derive from catecholamines with free OH oup in the 3-position (*cf* Jonsson 1966). Thus, DA-4-*O*-glucuronide is present in the ster insuloma.

The second unidentified substance moved considerably faster on paper chromatograms an the DA-4-O-glucuronide. When exposed t formaldehyde gas on paper or when ated with formaldehyde and hydrogen peroxide according to Hess and Udenfriend (1959)

Sigma kit no 325 Three determinations on single tumours gave 1 325 1 320, and F Fishman units per gram tissue.

The enzymatic hydrolysis and direct extraction yielded considerably higher values dopamine in this experiment than those found with acid hydrolysis and the correspondi control determination. These two experiments can not however be directly compar Firstly the process involved in the acid hydrolysis has been shown to cause a certain los catecholamines. Secondly a contributing reason for this discrepancy may derive from t fact that there have been 18 passages of the tumour between these two studies.

Comparative studies using synthetic 4-O-(β-D-glucopyranosiduronic acid)-dopamine (D 4-O-glucuronide). The DA-4-O-glucuronide was synthesized (Hansson and Rosengren 19 and this synthetic compound was used to establish the presence of the substance in insuloma tissue

Extracts from 3 tumours were run on paper as described above using the synth DA-4-O-glucuronide as reference The spots were visualized using the formaldehyde pro dure. In all 3 cases the slow-moving substance from the insuloma had the same Rf v (0 10) and fluorescence characteristics as the reference substance when determined in spectrophotofluorometer after elution of the fluorophores with 0.1 M HCl. When the s thetic DA-4-O-glucuronide was dissolved in 0.1 M HCl and then treated with formald and hydrogen peroxide according to Hess and Udenfriend it displayed the same fluoresc characteristics as those of the material isolated from the insuloma. Fluorescence spectra tained from the 2 substances at pH 1 7 and 11 were also identical. The emission maxi remained unchanged but a pronounced wave-length shift from 350 nm at pH 1 to 37 at pH 7 occurred in the excitation maximum with a concomitant sharp increase in flu cence intensity A slight decrease of the excitation maximum and a lower intensity registered at pH 11

Demonstration of 3-methoxytyramine and its glucuronide Tissue extracts were prep and purified on the cation exchange resin (cf Material and Methods). 25 ml 1.2 M HCl passed through the column and the effluent was discarded Further elution was perfor with 20 ml 2 M HCl and the whole eluate was evaporated to dryness. The residue was pared for GC MS as above The mass spectrum revealed the presence of 3-methoxytyra (Fig. 1).

Extracts from insulomas were run on paper and the fast-moving spot (Rf 0.28) was el The eluate was incubated with Type H 2 crude extract at 37°C and ±0 C respectively Incubates were purified and prepared for GC MS as described for 3-methoxytyramine GC MS analyses showed the presence of this substance only in the extract from the inc tion at +37°C.

To get an estimate of the amount of 3-methoxytyramine-4-O glucuronide present in insuloma, 2 tumours were homogenized in buffer for direct extraction and for incub in the presence of Type H-2 extract with and without phosphate ions following the proce described above for dopamine glucuronide The content of 3-methoxytyramine was d mined according to Carlsson and Waldeck (1964) Direct extraction gave no signif amount of 3-methoxytyramine according to this fluorometric method whereas the enzy hydrolysis gave 7 6 and 7.2 μg 3-methoxytyramine/g tumour tissue

Discussion

-lier studies using the Falck Hillarp histochemical method, have shown that exposure to formaldehyde gas gives an intense blue fluorescence in hamster insuloma cells (Cegrell *et al.* 1969). The spectral characteristics were different from those displayed by the fluorophores of all catechol and indole derivatives which have been studied according to this method in tissues and model systems. This indicated the presence in the insuloma of one or several unidentified fluorogenic compounds whose formaldehyde fluorophores had an emission peak at considerably lower wave lengths than biogenic catechol and indole derivatives studied so far.

In the present work, paper chromatographic analyses (phenol −0.1 M HCl, 9 1) have demonstrated 2 compounds in the insuloma tissue with Rf values 0.10 and 0.28 respectively that develop a blue fluorescence when the paper is exposed to formaldehyde gas under the reaction conditions used in the histochemical formaldehyde method.

Acid hydrolysis of the slow moving compound and of homogenized tumour tissue result in the formation of dopamine. The substance should thus be a dopamine derivative, possibly the O-sulfate or the O-glucuronide. Incubation of tumour homogenates in the presence of glucuronidase and sulfatase demonstrated considerable formation of dopamine due to the β-glucuronidase activity The magnitude of this amine formation did not change upon addition of the sulfatase. The compound present in the tumour should thus be a dopamine glucuronide.

This dopamine-glucuronide could be a mono- or a diglucuronide though from a biological point of view it seems highly improbable that the diglucuronide is formed. To further elucidate this problem it was of importance to compare the properties of the substance in hamster insuloma with those of synthetic dopamine-glucuronide, preferable 4-O-(β-D-glucopyranosiduronic acid)-dopamine, (DA-4-O-glucuronide). To our knowledge a synthesis of this compound has not hitherto been described. A synthetic method had therefore to be worked out (Hansson and Rosengren 1976). Paper chromatograms showed that the slow-moving substance from tumour extracts had the same Rf value as the synthetic substance and moreover that the formaldehyde-induced fluorophores of both substances had the same fluorescence characteristics Convincing evidence that the dopamine-glucuronide isolated from insuloma tissue is not diglucuronide but monoglucuronide substituted in the 4-position was obtained by eluting both this and the synthetic compound from paper chromatograms and inducing fluorophore formation with formaldehyde and hydrogen peroxide in solution according to Hess and Udenfriend (1959). The spectral characteristics of the two fluorophores recorded at different pH values revealed a wave-length shift of the excitation maximum and a pH dependent fluorescence intensity These phenomena are significant for fluorophores having a quinoidal structure implying that they derive from catecholamines with free OH-group in the 3-position (*cf* Jonsson 1966). Thus, DA-4-O-glucuronide is present in the hamster insuloma.

The second unidentified substance moved considerably faster on paper chromatograms than the DA-4-O-glucuronide. When exposed to formaldehyde gas on paper or when treated with formaldehyde and hydrogen peroxide according to Hess and Udenfriend (1959)

It displayed the same fluorescence characteristics as the DA-4-O-glucuronide at p yielded no dopamine upon acid hydrolysis. Since the occurrence of 3-methoxytyr the tumours was established in the present work, it appeared reasonable to assume substance could be 3-methoxytyramine-4-O-glucuronide In fact incubation wi curonidase or acid hydrolysis resulted in the formation of a substance that in c GC MS was unequivocally identified as 3-methoxytyramine

Thus, there are at least two conjugated catechol derivatives in this islet cell tumc of these the dopamine-glucuronide, is present in extremely high concentration, gre hitherto found in any mammalian tissue Both can be estimated by the Hess and Ud (1959) fluorescence reaction but this does not distinguish between them. Administr L-dopa or the dopa-decarboxylase inhibitor NSD 1015 produces, within 2 h or le siderable changes in the concentrations of these two substances as measured by the cence reaction There is thus a rapid turnover of L-dopa and formation of one or the glucuronides in the tumour tissue

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References

ANTON A. H and D F SAYRE, The distribution of dopamine and dopa in various animals and for their determination i diverse biological material. *J Pharmacol xp. Ther* 1964 *145* 326-

AXELSSON S., L. CEGRELL and A M ROSENGREN, Tyrosine hydroxylase activity in a transplant cell tumour of golden hamster *Experientia* (Basel) 1970 *26* 998–999

BJÖRKLUND A., B FALCK and CH. OWMAN, Fluorescence microscopic nd microspectrofl techniques for the cellular localization and characterization of biogenic amines. In. *Methods of tive and Diagnostic Endocrinology* (Ed. S A. Berson), V l. I *The Thyroid and Biogenic Am* J E. Rall and I J Kopi). North-Holland Publ. Comp., Amsterdam 1972. pp. 318–368.

CARLSSON A. and B WALDECK A method for the fluorimetric determination of 3-methoxyty tissues and the occurrence of this amine in brain. *Scand. J l n. Lab. Invest* 1964 *16* 133–13

CEGRELL, L., B F LCK and A. M ROSENGREN, Catech l and indole derivatives i a transplant cell tumour of the golden hamster *A ta physiol scand.* 1969 a. *77* 23–?7

CEGRELL, L., B. FALCK and A. M ROSENGREN, Dopa-decarboxylase and monoamine oxidase ac a transplantable islet cell tumour of the golden hamster *Experientia* (Basel) 1969 b. *25* 969–97

COSTA, E., A. R. GREEN S. H KOSLOW H. F LEFEVRE, A. V REVUELTA and C. WANG, Dopa norepinephrine in noradrenergic a ons. A tudy *in h* of thei precurso product relationship fragmentography and radiochemistry *Pharmacol. Rev* 1972. *24* 178.

FALCK, B Observations on the possibilities of the cellular localization of monoamines by flu method. *Acta physiol. scand* 1962. *56* Suppl. 197

FALCK B., N Å HILLARP G THIEME and A TORP Fluorescence of catechol amines and rela pounds condensed with formaldehyde. *J Histochem Cytochem.* 1962. *10* 348–354

HANSSON C. and E. ROSENGREN, Synthesis of 4-O-(β-D-glucopyranosiduronic acid)-dopamine. *A scand.* 1976. In press.

HESS, S. M nd S. UDENFRIEND, A fluorometric proced re for the measurement of tryptamine i *J Pharmacol exp. Ther* 1959 *127* 175–177

JOHNSON, G Fluorescence studies on some 6,7-substituted 3 4-d hydroisoquinolines formed from 3-h tyramine (dopamine) and formaldehyde. *A ta chem scand.* 1966. *20* 2755–2762.

KARTNIG, TH. and O WROSCHAUER, Eine Möglichkeit zur Identifizierung von Zuckern aus l Mengen von Glykosiden oder aus Zuckergemischen *J Chromatogr* 1971 *61* 375–377

KIRKMAN, H A preliminary report concerning tumors observed i Syrian hamsters. *Stanford M* 1962. *20*. 163–165

TAIT J M S T W SHANNON and A. G HAR ISON, The structure of substituted C_7 ions from derivatives at the ppearance potential threshold. *J Amer hem Soc* 1962. *84* 4-8.

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Actomyosin ATPase, Myokinase, CPK and LDH in Human Fast and Slow Twitch Muscle Fibres

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Abstract

…ORSTENSSON, A., B. SJÖDIN, P. TESCH and J. KARLSSON. *Actomyosin ATPase, myokinase, CPK and LDH in human fast and slow twitch muscle fibres.* Acta physiol. scand. 1977. 99. 225–229.

…e enzyme activities of Mg^{++} stimulated ATPase, creatine phosphokinase (CPK), myokinase (MK) …d lactate dehydrogenase (LDH) were determined in pooled fast twitch (FT) and slow twitch (ST) human …eletal muscle fibres, dissected out from freeze-dried muscle biopsy material. All enzymes investigated …monstrated higher activities in FT fibres. The ratio in enzyme activity between fibre types was greatest … Mg^{++} stimulated ATPase (3:1) and smallest for CPK (1.3:1). In addition, the isozyme patterns of …PK, MK and LDH were studied by means of isoelectric focusing (CPK and MK) and discelectrophoresis …DH). A difference was observed between fibre types with respect to the isozyme distribution of MK and …DH, whereas the CPK isozyme pattern was similar in both fibre types. These results on separated human …T and ST fibres are essentially in conformity with what has earlier been indicated from experiments on …ved muscle homogenates.

…ost human skeletal muscles contain a mixture of fast twitch (FT) and slow twitch (ST) muscle fibres. The relative distribution of these two main fibre types may however vary considerably (Gollnick *et al.* 1972 and 1974). By biochemical analyses of muscle homogenates with a wide range of fibre compositions it has been possible to demonstrate muscle fibre dependency of several enzymes involved in the energy yielding processes of the muscle (Gollnick *et al.* 1974, Essén *et al.* 1975).

Such studies have formed the basis for the metabolic profiles assigned the two main human muscle fibre types, a high glycolytic capacity for FT fibres and a high oxidative potential for ST fibres. Also some of the enzymes involved in the immediate ATP turn-over in connection with muscle contraction have been demonstrated to have activities that varied with muscle fibre composition. Thus, muscle samples with a high proportion FT fibres showed a higher activity of myosin ATPase (Taylor *et al.* 1974), Mg^{++} stimulated ATPase, and myokinase (MK) (Thorstensson *et al.* 1976 a). Furthermore, in the case of enzymes like lactate dehydrogenase (LDH) and MK, muscle fibre type related differences have been observed also in isozyme distribution (Karlsson *et al.* 1974, Thorstensson *et al.* 1976 b).

Similar relationships have been reported for creatine phosphokinase (CPK) in muscle (Sherwin *et al* 1967) but could not be confirmed in mixed human muscle homo (Thorstensson *et al* 1976 a and b)

Recently however a technique has been described to dissect out and identify and ST fibres from freeze-dried muscle biopsy material (Essén *et al.* 1975). This makes it possible to perform biochemical analyses on pure samples of the two m types in human skeletal muscle FT and ST fibres.

The purpose of the present investigation was to use this technique to compare the and isozyme patterns of Mg stimulated ATPase, CPK, MK, and LDH in hu and ST muscle fibres and thereby examine the validity of results obtained in exp on mixed muscle homogenates.

Material and methods

M terial. Muscle biopsies were obtained at rest with the needle biopsy technique (Bergstrom 1962 vastus lateralis of two habitually active healthy male subjects (2 and 25 yrs of age).

Muscle fibre pr paration. The method fo preparation of single muscle f bres was essentially to Essén *et al* (1975). Briefly dissection of f bres was performed from freeze-dried material section microscope i a oom with constant temperature and h midity (20°C and 35 , res Fibre classification as FT or ST was done on the cut off ends of each fibre, which ere stained fibrillar ATPase (Padykula and Herma 1955) fter preincubation at pH 10.3 The remaining p the f bres were pooled together according to fibre type. The total umber of fibres dissected 1 818 FT and 1 804 ST f bres, respectively Before further analyses the pooled muscle f bres ogenized by sonicatio 200 / l 0.5 M KCl

Biochemical analyses. The enzyme ctivities were determined fluorometrically using NA coupled reactions accordi g to Lowry (Lowry and Passonneau 1972). The enzymes under inv were Mg^{++} stimulated ATPase (E.C. 3 6.1 4), creatine phosphokinase, CPK (E.C. 2.7 3.2.), m MK (E.C. 2.7 4.3), and lactat dehydrogenase, LDH (E.C. 1 1 1.27). The test procedures were as those earlier used fo the corresponding enzyme activity analyses on whole muscle homogen methodological errors f r these determinations were found t be 8 % (Mg^{++} stimulated ATPase) (CPK and MK), respectively (Thorstensso unpubl results). The effect of freeze-drying on the these enzymes was investigated in separate study No decrease i enzyme activities was found freeze-drying procedure. The dry weight corresponded t 25 % of the wet weight of the samples

The protein content of the homogenized f bres was determined with the fol reaction acc Lowry (Lowry *et al.* 1951).

Isozyme separation The techniques f isozyme nalyses were the same as earlier described muscle homogenates (Karlsson *et al* 1974 Thorstensson *et al.* 1976 b). LDH isozymes ere sep con entional discelectrophoresis, whereas isoelectric focusing in polyacrylamide and granulated gels were used for CPK and MK isozyme separation, respectively The isozymes were detected by zymogram techniques and the relative tensity of the stained band was evaluated by densitomet ing. (For detailed inf rmation, see Karlsso et al 1974 Sjödin et al 1976, and Thorstensson et al.

Results and discussion

Enzyme activities All enzymes investigated demonstrated higher levels of activity FT than in the ST fibres (Table I)

The greatest difference was seen for Mg stimulated ATPase. The activity of this which is regarded to be controlling the contractile activity in muscle (Perry 197 about 3 times higher in the FT fibres. A similar relationship was from expe

...e 1. Enzyme activities in pooled fast twitch (FT) and slow twitch (ST) skeletal muscle fibres dissected out from freeze-dried human biopsy material.

...yme	FT	ST	FT/ST ratio
Ca++ stimulated ATPase	0.84	0.30	2.8 : 1
...atine phosphokinase	16.6	13.1	1.3 : 1
...okinase	12.1	6.6	1.8 : 1
LDH lactate→pyruvate	3.66	1.45	2.5 : 1
LDH pyruvate→lactate	3.68	[illegible]	2.0 : 1

...ies are in moles $\cdot 10^{-6} \cdot$ g protein^{-1} $\cdot$ min^{-1}. LDH is short for lactate dehydrogenase.

...mixed muscle homogenates, although not until after a period of physical conditioning ...horstensson *et al.* 1976). The ATPase activity of purified human myosin has been shown ...be 2.3 times higher in FT muscle fibres (Essén *et al.* 1975). The finding was also in con...mity with results obtained from animal skeletal muscle preparations, where a high ...ortion dark staining (FT) fibres in the muscle coincided with a high actomyosin ATPase ...ty (Guth and Samaha 1969).

...e MK activity in FT fibres was about twice that in ST fibres. This ratio between fibre ...s in MK activity was somewhat lower than could be obtained by extrapolating the ...ession lines of earlier obtained correlations between % FT and MK activity (Thors...son *et al.* 1976 a, Komi *et al.* to be published). However the general tendency of a ...er MK activity in FT muscle fibres found in those studies was confirmed.

...higher activity of CPK in FT fibres has been indicated in animal experiments (Burke ... Edgerton 1975). A similar relationship for CPK was not found in human skeletal ...cle (Thorstensson *et al.* 1976 a). In the present study the difference in CPK activity ...ween FT and ST fibres was relatively small, about 30 %, which could explain the difficulty ...obtain any correlation between enzyme activity and muscle fibre type in the above men...ned experiments on mixed human muscle homogenates.

LDH activity was higher by a factor of 2.2.5 in FT as compared to ST muscle fibres ...spective in which direction the enzyme activity was determined. This was in agreement ...h earlier observations in human skeletal muscle (for refs. see Sjödin 1976). Moreover ...could be calculated that the LDH activity in the forward direction (pyruvate→lactate) ...er the backward direction (lactate→pyruvate) was about the same in both fibre types ...6.1 and 1.9 : 1 in FT and ST fibres, respectively).

...zyme distribution. The isozyme pattern of CPK after isoelectric focusing showed no ...fference between FT and ST muscle fibres. In both cases 2 major bands were visible with ...oelectric points (pIs) of about 7.4 and 7.0, respectively. The most alkaline band constituted ...both instances approximately 70 % of total stain density. These figures were in good ...cordance with values earlier obtained for mixed muscle homogenates (Thorstensson *et al.* 1976 b).

Also the separation of MK isozymes gave results in agreement with what had previously ...een postulated from whole biopsy analysis (Thorstensson *et al.* 1976 b). Thus, two MK ...ands were observed in the alkaline region of the gel with pIs of approximately 10.0 (MK 1) ...nd 9.0 (MK 2), respectively (Fig. 1). The MK 1 band predominated in both fibre types,

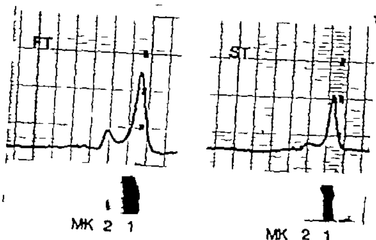


Fig. 1. The isozyme patter
myokinase (MK) in pooled hu
fast twitch (FT) and slow t
(ST) muscle fibres after isoele
focusing. The stained prot
the corresponding curves
densitometric scanning are sh

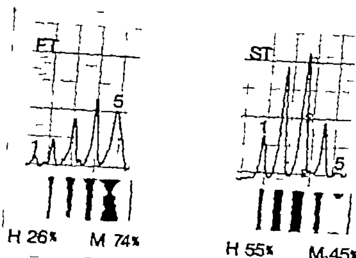


Fig. 2. The isozyme pattern
lactate dehydrogenase (LDH)
pooled human fast twitch (F
and slow twitch (ST) mus
fibres after discelectrophores
The results of densitometric sca
ning are shown as well as the c
culated portion of H (heart) a
M (muscle) type of LDH subu

but the relative contribution of this isozyme to total stain density was higher in ST fibr (87%) as compared to FT fibres (73%). The faint MK 2 band in ST fibres was hard visible upon reproduction (Fig. 1).

The LDH isozyme pattern demonstrated that the skeletal muscle specific subunit, th M subunit (for refs. see Sjödin 1976) predominated in the FT fibres, whereas the hea muscle specific subunit, the H subunit, appeared with a higher relative stain density in th ST fibres (Fig. 2). These findings confirmed observations earlier made by Sjödin (197 and would support the suggestion of a more pronounced anaerobic glycolytic potential i the FT fibres, and a higher capacity for lactate combustion in the ST fibres.

In summary, the present results obtained on pooled single muscle fibres essentially co firmed what had earlier been deduced from experiments on mixed human skeletal musc homogenates. Thus, the conclusions made in those studies about a metabolic profile of FT muscle fibres that is more in favour of fast contraction and rapid ATP replenishment as compared to ST muscle fibres, were further strengthened.

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References

ergström, J., Muscle electrolytes in man. *Scand. J. clin. Lab. Invest.* 1962. Suppl. 68.

urke, R. E. and V. R. Edgerton, Motor unit properties and selective involvement in movement. *Exercise and Sport Sci. Rev.* 1975. 3. 31–81.

ssén, B., E. Jansson, J. Henriksson, A. W. Taylor and B. Saltin, Metabolic characteristics of fibre types in human skeletal muscle. *Acta physiol. scand.* 1975. 95. 153–165.

ollnick, P. D., R. B. Armstrong, C. W. Saubert IV, K. Piehl and B. Saltin, Enzyme activity and fibre composition in skeletal muscle of untrained and trained men. *J. appl. Physiol.* 1972. 33. 312–319.

ollnick, P. D., B. Sjödin, J. Karlsson, E. Jansson and B. Saltin, Human soleus muscle: a comparison of fiber composition and enzyme activities with other leg muscles. *Pflügers Arch. ges. Physiol.* 1974. 348. 247–255.

uth, L. and F. J. Samaha, *Qualitative differences between actomyosin ATPase of slow and fast mammalian muscle. Exp. Neurol.* 1969. 25. 138–152.

arlsson, J., K. Frith, B. Sjödin, P. D. Gollnick and B. Saltin, Distribution of LDH isozymes in human skeletal muscle. *Scand. J. clin. Lab. Invest.* 1974. 33. 307–312.

owry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the folin phenol reagent. *J. biol. Chem.* 1951. 193. 265–275.

owry, O. H. and J. V. Passonneau, *A flexible system of enzymatic analysis.* Academic Press, New York 1972.

adykula, H. A. and E. Herman, The specificity of the histochemical method for adenosine triphosphatase. *J. Histochem. Cytochem.* 1955. 3. 170–195.

erry, S. V. Calcium ions and the function of the contractile proteins of muscle. *Biochem. Soc. Symp.* 1974. 39. 115–132.

herwin, A. L., G. R. Sobel and M. M. Elhilali, Fluorescence technique to demonstrate creatine phosphokinase isozymes. *Clin. chim. Acta* 1967. 17. 245–249.

jödin, B. Lactate dehydrogenase in human skeletal muscle. *Acta physiol. scand.* 1976. Suppl. 436.

jödin, B., A. Thorstensson, K. Frith and J. Karlsson, Effects of physical training on LDH activity and LDH isozyme pattern in human skeletal muscle. *Acta physiol. scand.* 1976. 97. 150–157.

aylor, A. W., B. Essén, and B. Saltin, Myosin ATPase in skeletal muscle of healthy men. *Acta physiol. scand.* 1974. 91. 568–570.

horstensson, A., B. Hultén, W. von Döbeln and J. Karlsson, Effect of strength training on enzyme activities and fibre characteristics in human skeletal muscle. *Acta physiol. scand.* 1976. 96. 392–398.

horstensson, A., K. Elwin, B. Sjödin and J. Karlsson, Isozymes of creatine phosphokinase and myokinase in human heart and skeletal muscle. *Scand. J. clin. Lab. Invest.* In press.

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Lactate in Fast and Slow Twitch Skeletal Muscle Fibres of Man during Isometric Contraction

By

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Abstract

TESCH P and J KARLSSON *Lactate in fast and slow twitch skeletal muscle fibre during isometric contraction* Acta physiol scand. 1977 99 230–236.

Concentrations of lactate in fast and slow twitch f bres, respectively were determined in m femoris fter sustained contractions t 25%, 50%, and 75% f max mal oluntary isometric c (MVC) until exhaustion as well as after i terrupted exercises at 25 and 50 MVC. Maxim concentrations were only found at 50% of MVC perf rmed to exhaustion. Lactate concentr higher in slow twitch (ST) f bres at 50% MVC compared to in ST fibres at 25 MVC, and hig twitch (FT) f bres at 50% MVC compared t in FT fibres at 75% MVC. After short t me isometr (i.e 75% t exhaustion and 50% nd 25% performed for the same period of t me as 75 MV concentration, expressed as lactate ratio (lact te concentration in FT fibres/lactate concentrat fibres) was found to be positively correlated to percent FT f bres (r = 0 89). Lactate ratio range i.e at onset of isometric exercise, lactate concentratio increase was faster n ST fibres in the mus ST fibres and faster in FT f bres when the muscle was rich in FT f bres.

With exhaustive isometric exercise maximal values for ATP and creatine phosphat tion as well as lactate accumulation are observed at tensions corresponding to 30- maximal voluntary contraction (MVC) (Karlsson and Ollander 1972, Karlsson *et a* Similar high values are obtained during short-term maximal dynamic (bicycle) work son and Saltin 1970 Karlsson 1971) At lower (10–25% MVC) and higher (75% exhaustive isometric tensions "submaximal" values for phosphagen depletion and accumulation are observed (Karlsson and Ollander 1972) At tensions less than 20 mainly slow twitch (ST) fibres are depleted on their glycogen (Gollnick *et al* 1974 a) ing to estimation of glycogen content in individual muscle fibres, on the basis of PAS intensity (Gollnick *et al* 1972), whereas mainly fast twitch (FT) fibres are depl glycogen at tensions in excess of 25% MVC If the selective glycogen depletion r represent muscle fibre recruitment it would indicate that at light tensions only ST fib recruited, while at heavier tensions only FT fibres are recruited Gydikov (1975) has re that at light (30–40% MVC) isometric muscle tensions only slow motor units are act First at tensions in excess of 30–40% MVC fast motor units are recruited but slow units are still discharging.

Table 1. Individual anthropological and physiological data.

Subject	Age (yrs)	Height (cm)	Weight (kg)	Fibre comp (% FT)	MVC (kp)	Max. performance time, min MVC		
						75	50	25
	25	179	71	68	202	0.46	0.94	3.40
	21	189	83	58	198	0.70	1.42	5.90
	19	185	78	45	246	0.90	1.16	4.00

As great variations in lactate accumulation are present within a whole biopsy specimen (Karlsson 1971) it seems reasonable to suggest that exhaustion at low as well as high tensions are depending on high lactate concentrations and phosphagen depletions in recruited motor units. The present study was therefore undertaken to evaluate lactate concentration in individual muscle fibre types at different isometric tensions performed either submaximally or to exhaustion.

Subjects, procedure and methods

Healthy male students of physical education were employed as subjects. Some of their characteristics are summarized in Table 1. Isometric exercise was performed in a chair constructed for leg exercise (Karlsson and Ollander 1972). By pressing the feet in a sitting position, against an immovable bar mainly the thigh muscles are activated (Karlsson et al. 1975). The subjects trained in the chair 2–3 days prior to the expts. to learn the technique and to get acquainted with the experimental set up. The day before the expts. maximal voluntary contraction (MVC) was determined. The final expts. were conducted over two days. 75, 50, and 25% of MVC were calculated for each subject and performed to exhaustion. The rest period between the separate bouts was at least 2 h. In a second set of expts. the subjects were studied at 50% and 25% of MVC respectively but the expts. were interrupted at the time corresponding to exhaustion for 75% MVC. In Fig. 1 the protocol of the expts. is illustrated.

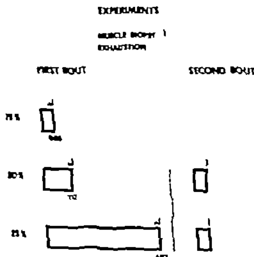


Fig. 1. Experimental protocol. Mean time (min) are expressed below the squares.

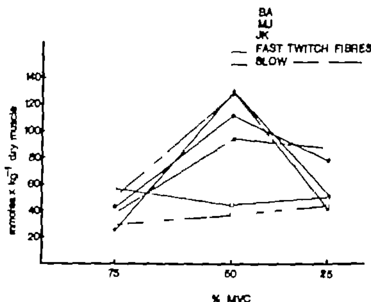


Fig. 2. Lactate concentr fast twitch (FT) and slo (ST) muscle fibres, resp at exhaustion after 75 50 MVC leg muscle contract

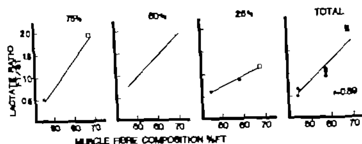
Muscle biopsies (Bergström 1962) were taken from the lateral portion of the quadriceps femor before the exercise tests for determination of muscle f bre composition according to Gollnick et Immediately after termination of exercise (within 3–5 s) with the subject still sitting in the chair biopsies were taken for determination of muscle f bre lactate concentration. The specimens ere ately frozen in liquid nitrogen and stored at −80°C for later analysis (Karlsson 1971)

Analysis for lactate concentration on individual muscle fibre groups were made on muscle f bres from freeze dried biopsies (Essén *et al* 1975). The freeze dried biopsy specimen were then vacuum at −80°C before nalyses. After dissecting at 21°C with a humid ty of 30 ., the fib identified by means of myofibrillar ATPase staining (for ref see Essén *et al*. 1975). Identified ST fi FT f bres, respectively were then pooled and weighed. Fluorometric techniques (Karlsson 1971) h been used to determine lactat concentration in 30–60 slow and fast twitch f bres, respectively p each sample. The weight of the f bres independent of fibre type, were n each determination bet ee 150 μg. When analysing f bres from the same freeze dried biopsy specimen at separate days (week) the coefficient of variation was 14 % as compared to a coefficient of variation of 6 %, bet different parts of the biopsy the same day

Results

The pattern of maximal endurance time in relation to relative force was similar t earlier has been reported (Monod and Sherrer 1957 Rohmert 1960, Humphreys an 1963 Karlsson and Ollander 1972, and Karlsson *et al* 1975) The mean performan at 75 50 and 25% MVC, respectively averaged 0 55 (range 0 46–0 70) min, 1 17 0.94–1 42) min and 4 63 (range 3 40–5 90) min

With exhaustive exercise mean lactate concentration was highest after 50% M ST as well as FT fibres, 86 (range 37–128) and 96 (range 44–130) mmoles kg⁻ dry respectively This was true for 2 of 3 subjects studied. One subject demonstrated a mately the same lactate concentration after exhaustive 75% and 25% MVC, respe 75 and 25% MVC resulted in a mean lactate concentration of 41 (FT fibres) and fibres), and 60 (FT fibres) and 58 (ST fibres) mmoles kg dry weight, respectively vidual values are presented in Fig. 2. The values after 50% MVC should expressed as m



Lactate ratio, ... lactate concentrations in FT fibres versus lactate concentrations in ST ... expressed in relation to muscle fibre composition (% FT) after 75, 50 and 25% MVC performed for ... period of time as 75 ... MVC to exhaustion, $p<0.001$, $y=0.04$ ... -1.34.

... wet weight, reach about 20–22 (Karlsson 1971). Lactate concentration obtained at ... ation for the 3 different tensions are in close agreement with those earlier obtained ... hole biopsy specimens (Karlsson and Ollander 1972, and Karlsson *et al.* 1975).

After "submaximal" 50% and 25% MVC lactate concentration averaged 35 (FT fibres), 32 (ST fibres), and 29 (FT fibres), and 41 (ST fibres) mmol · kg⁻ dry weight. These concentrations were consequently similar to the values obtained at 75% MVC and exhaustion indicating a similar rate of initial muscle fibre lactate accumulation irrespective 25–50 75% MVC.

Muscle fibre composition for the 3 subjects varied (Table I). Thus one subject (BA) had % FT fibres and another (JK) 45% FT fibres. If lactate content was expressed as lactate concentration in FT fibres versus ST fibres (lactate FT/ST ratio Fig. 3) subject BA had ... of 1.93 after exhaustive short time exercise (75% MVC) while the corresponding ratio ... subject JK reached 0.51. After "submaximal" (50% MVC) short time exercise lactate ratio for subject BA was 1.99 and for subject JK 0.88. For subject MJ (58% FT fibres) the corresponding values were 1.07 and 1.02, respectively. After "submaximal" (25% MVC) short time exercise the lactate ratio for BA, MJ and JK were 1.15, 0.83 and 0.63 respectively. When the 50 and 25 ... MVC tensions were maintained until exhaustion, the lactate FT/ST ratio approached unity for the two extremes BA and JK, respectively (Fig. 4).

Discussion

Lactate concentration differences between muscle and blood in man immediately after short time (6–10 min) submaximal or maximal bicycle exercise suggest that a fast translocation of lactate do not take place neither between lactate producing fibres and the extracellular space nor the extracellular space and nonrecruited fibres (Karlsson 1971 and Jorfeldt *et al.* 1974). In fact after short time maximal exercise 6–10 min has to lapse before an equilibrium is

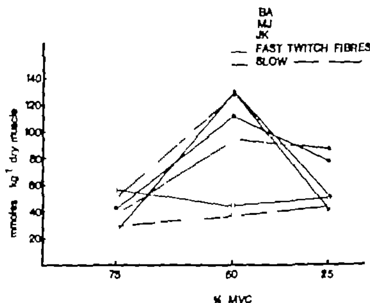


Fig. 2. Lactate concentration in fast twitch (FT) and slow twitch (ST) muscle fibres, respectively at exhaustion after 75, 50 and 25% MVC leg muscle contraction

Muscle biopsies (Bergström 1962) were taken from the lateral portion of the quadriceps femoris before the exercise tests for determination of muscle fibre composition according to Gollnick *et al.* Immediately after termination of exercise (within 3–5 s) with the subject still sitting in the chair, biopsies were taken for determination of muscle fibre lactate concentration. The specimens were immediately frozen in liquid nitrogen and stored at −80°C for later analysis (Karlsson 1971).

Analysis for lactate concentration on individual muscle fibre groups were made on muscle fibres from freeze dried biopsies (Essén *et al.* 1975). The freeze dried biopsy specimen were then stored in vacuum at −80°C before analyses. After dissecting at 1°C with a humidity of 30 %, the fibres were identified by means of myofibrillar ATPase staining (for ref. see Essén *et al.* 1975). Identified ST and FT fibres, respectively, were then pooled and weighed. Fluorometric techniques (Karlsson 1971) have been used to determine lactate concentration in 30–60 slow and fast twitch fibres, respectively, per each sample. The weight of the fibres, independent of fibre type, were in each determination between ... 150 μg. When analysing fibres from the same freeze dried biopsy specimen at separate days (within a week) the coefficient of variation was 14 % as compared to a coefficient of variation of 6 % when analysing different parts of the biopsy the same day.

Results

The pattern of maximal endurance time in relation to relative force was similar to what earlier has been reported (Monod and Sherrer 1957, Rohmert 1960, Humphreys and Lind 1963, Karlsson and Ollander 1972, and Karlsson *et al.* 1975). The mean performance time at 75, 50 and 25% MVC, respectively, averaged 0.55 (range 0.46–0.70) min, 1.17 (range 0.94–1.42) min and 4.63 (range 3.40–5.90) min.

With exhaustive exercise mean lactate concentration was highest after 50% MVC in ST as well as FT fibres, 86 (range 37–128) and 96 (range 44–130) mmoles · kg⁻¹ dry weight, respectively. This was true for 2 of 3 subjects studied. One subject demonstrated approximately the same lactate concentration after exhaustive 75% and 25% MVC, respectively. 75 and 25% MVC resulted in a mean lactate concentration of 41 (FT fibres) and 4... (ST fibres), and 60 (FT fibres) and 58 (ST fibres) mmoles · kg⁻¹ dry weight, respectively. Individual values are presented in Fig. 2. The values after 50 % MVC should, expressed as mm...

been suggested that at short time maximal exercise (below 1 min) neuromuscular fatigue take place and is most marked in high threshold motor units, while contrac... fatigue affects low threshold motor units (Stephens and Taylor 1972). Another determinative for performance time and exhaustion could possibly be the rate of ...ture rise in activated muscle fibres. It was recently pointed out (Bolstand and Ers... 76) that heat production was greater in FT fibres compared to ST fibres and that the temperature rise was twice as high in muscles rich in FT fibres (m. biceps brachii) ...pared to muscles rich in ST fibres (m. soleus).

...ubject with a high percent FT fibres had a higher lactate concentration in the FT ...xpressed as lactate ratio FT/ST as compared to the subject with a low percent FT. This observation was made after exercise at different tensions, exhaustive or not, performance time corresponding to about 0.5 min. This means that the subject rich ...twitch fibres (JK) at all tensions studied after 0.5 min of exercise demonstrated ...lactates in the ST fibres (Fig. 3).

FT/ST lactate ratio in the subject with a low percent FT fibres in his muscle seems to ...dict the conclusion by Gollnick *et al.* 1974 a, that at sustained contractions at low ...ric tensions there is a major reliance upon ST fibres and above that level a primary ...dence upon FT fibres. It is not at present possible to causally explain this difference. ...fect of individual variation in muscle fibre populations was, however not considered ...llnick *et al.* 1974 a, as no extremes in that respect were included in the study (Karlsson, ...comm.). It has been reported that subjects with either a high percent ST or a high per... type II A (*i.e.* FT fibres with a high oxidative potential) do not fit into the glycogen ...tion pattern as suggested by Gollnick *et al.* 1974 a (Komi, pers. comm.).

...e activity of the enzyme lactate dehydrogenase (LDH) is lower in muscles rich in ST ...compared to muscles rich in FT fibres (Gollnick *et al.* 1974 b and Sjödin 1976). ...separating the different LDH isozymes (Karlsson *et al.* 1974) it is evident, that in ...ion to higher LDH activity the FT fibres contain a higher percent of the LDH isozyme, ...-5 or the most skeletal muscle specific isozyme as compared to ST fibres, which are ...in LDH 1 or the heart muscle specific isozyme. Moreover it has been suggested (Baba ...Sharma 1971) that LDH-1 and LDH 2 are localized in mitochondria, which are in...ed in aerobic metabolism while LDH-5 is found in sarcoplasmic reticulum and should ...nvolved in the anaerobic glycolysis.

...seems reasonable to assume that similar properties of the different muscle fibre types ...t affect lactate metabolism as indicated by Sjödin (1976). The present results in subject ...cannot, however be interpreted otherwise than a more marked lactate production was ...ng place in his ST fibres, which would indicate a recruitment of predominantly ST fibres ...ll tensions studied.

References

...A, N and H. M. Sharma, Histochemistry of lactic dehydrogenase in heart and pectoralis muscles of ... *J. Cell Biol.* 1971. *51*. 621–635.

...ström, J. Muscle electrolytes in man. *Scand. J. clin. Lab. Invest.* 1962. Suppl. 68.

...lund, C. and A. Ersland, Energy metabolism in different human skeletal muscles during voluntary ...metric contractions. 1976. T... be publ.

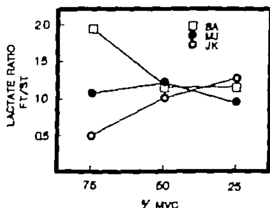


Fig. 4 Lactate ratio (for explanation see Fig
75 50 and 25 % MVC maintained to exhaustion

established (for ref see Karlsson 1971) It seems then reasonable to suggest that in this st
translocation processes for lactate only to a minor extent might obscure the pattern
lactate accumulation in the muscle related to lactate production. For the same reason tr
of the extracellular lactate contaminating the freeze dried muscle fibre samples ca
neglected as of significance for the interpretation of the results

The major finding in the present study was that lactate concentration in individual mu
fibres did not correspond to values as could be expected from glycogen depletion patt
in similar experimental situations (Gollnick *et al* 1974 a) High lactate concentrations
FT fibres were not found after exercise corresponding to heavy isometric tensions, and h
lactate concentrations were not found in ST fibres after light isometric tensions. Maxi
mean lactate concentration was obtained only after isometric, exhaustive exercise at 5
MVC and that was true for lactate concentration in both FT and ST fibres. One subj
did however differ from this pattern He had a muscle fibre composition with a predomin
of FT fibres (68 %) and also the shortest performance time at all tensions studied. The dif
ence in lactate concentration between the main muscle fibre types was not significan
any tension Thus, as lactate concentration at exhaustive exercise at 75 and 25 % M
respectively did not reach maximal values in any fibre type, it seems reasonable to sug
that lactate concentration in recruited fibres accordingly cannot explain exhaustion at th
tensions.

In the present study 30 or more individual fibres were pooled before analyses. Essén
Häggmark (1976) have demonstrated large variations in lactate content in individual mu
fibres picked from a pool of the same fibre type. This might indicate that exhaustion
marily are not related to the type of muscle fibre recruited but rather which fast or s
motor units are recruited

If a motor nerve recruited innervates ST fibres then their endurance capacity will be d
sive for performance (for ref see Close 1972 and Burke and Edgerton 1975). The metab
profile of the ST fibre population in human skeletal muscle speaks in favour for a m
pronounced endurance capacity of this main muscle fibre type (Gollnick *et al.* 197
Karlsson *et al.* 1974, and Essén *et al* 1975) and this has earlier been confirmed for isom
exercise (Hultén *et al* 1974) and recently for dynamic exercise (Thorstensson and Karls
1976).

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Effect of Changes in Blood Volume Distribution on Circulatory Variables and Plasma Renin Activity in Man

By

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Abstract

D S., J. CASTENFORS and L. E. LINDBLAD. *Effects of changes in blood volume distribution circulatory variables and plasma renin activity in man* Acta physiol. scand. 1977 237 245.

dy subjects the pressure around the lower body was changed to 40 mmHg bove (LBPP) and BNP) atmospheric, thereby altering the amount of blood pooled in the lower body Heart rate, al blood pressure, central venous pressure, cardiac output (dye dilution technique) and forearm w (venous occlusion plethysmography) were measured Plasma renin activity was determined adioimmunological method 6 subjects maintained relative circulatory steady state during LBNP caused significant decreases in central venous pressure (CVP), stroke volume and cardiac Q) with an unchanged mean arterial pressure (MAP) Heart rate (HR) increased significantly of total peripheral vascular resistance (TPVR) and regional vascular resistance (RVR) in the were significantly increased has measured 5–9 min after the onset of LBNP whereas plasma tivity (PRA) showed definite increase only after 19 min of LBNP stimulation. No correlation was etween the changes in PRA and TPVR or RVR. Increasing the pressure around the lower body resulted in slight but significant increase in MAP as well as significant but transient increase in le significant changes were found in HR, Q or TPVR. In the forearm decrease in RVR was rcted PRA was not significantly changed. The results demonstrate that peripheral vascular or and PRA are both influenced by changes in blood volume distribution, possibly elicited via ressor receptors sensitive to changes in central blood volume and/or CVP The results also that PRA does not play any significant part in the vasoconstriction during LBNP stimulation.

ords Blood volume, pressor receptors, cardiac output, heart rate, vascular resistance, plasma renin

rding to the classical concept, changes in the constrictor tone of the resistance vessels ediated via baroreceptors in the arterial high pressure circulation (Heymans and Neil).

owever it has been observed that even marked increase, of the order of 40 mmHg e transmural pressure of the carotid sinus, simulating considerable increase in MAP ts in only small changes in RVR (Bevegård and Shepherd 1966, Bevegård, Castenfors Lindblad, to be published).

n the other hand, during peripheral pooling of blood, causing decreased venous return, t is marked vasoconstriction, at least in the forearm, before or even in the absence of

BURKE, R E. and R. EDGERTON, Motor units properties and selective involvement in movement *and sport sciences reviews* 1975 3

CLOSE, R. I Dynamic properties of mammalian skeletal muscles. *Physiol. Rev* 1975, 52

ESSÉN B., E. JANSSON J HENRIKSSON A W TAYLOR, and B SALTIN, Metabolic characteristic types in human skeletal muscle. *Acta physiol scand* 1975, 95 153–165.

ESSÉN B. and T HÄGGMARK, Lactate concentration in type I and type II muscle fibres during contraction in man *Acta physiol scand*, 1975, 95 344 346.

GOLLNICK P D J KARLSSON K PIEHL and B. SALTIN, Selective glycogen depletion in skelet f bres of man following sustained contractions. *J Physiol.* (Lond.) 1974 a. 241 59–67

GOLLNICK, P D R B ARMSTRONG, C. W SAUBERT K. PIEHL and B. SALTIN, Enzyme activity composition in skeletal muscle of untrained and trained man. *J appl. Physiol* 1972, 33 3

GOLLNICK P D B SJÖDIN J KARLSSON, E. JANSSON and B. SALTIN, Human soleus muscle: A co of f ber composition and enzyme activities with other leg muscles. *Pflügers Arch. ges. Physio* 348 247 255

GYDIKOV A., Pattern of discharge of d fferent types of alpha motoneurons and motor un voluntary and reflex activity under normal physiological conditions. Fifth Intern Congres mechanics, Jyväskylä, Finland 1975

HULTÉN B., A THORSTENSSON, B. SJÖDIN and J KARLSSON, Relationship between isometric endu fibre types in human leg muscles. *Acta physiol. scand.* 1975, 93 135–138.

HUMPHREYS, P W and A. R. LIND, The blood flow through active and inactive muscles of th d ring sustained handgrip contractions. *J Physiol* (Lond) 1963 166, 120–135.

JORFELDT L., S. FORSBERG, A. JUHLIN-DANNFELT and J KARLSSON Relationship bet een lactate in skeletal muscle and release to the venous blood during bicycle exercise. Proc. of The Inte of Physiological Sciences, New Delhi 1974

KARLSSON, J and B. SALTIN Lactate ATP nd CP in working muscles during exhaustive exerci *J appl Physiol* 1970, 29 598–602.

KARLSSON J Lactate and phosphagen concentrations in working muscle of man. *Acta ph siol sc* Suppl 358.

KARLSSON J and B. OLLANDER Muscle metabolites with exhaustive static exercise of different *Acta physiol scand* 1972, 86 309–314

KARLSSON J C. FUNDERBURK, B. ESSÉN and A R. LIND Const tuents of h man muscle in isometri *J appl Physiol.* 1975 38 208–211

KARLSSON J K. FRITH B. SJÖDIN, P D GOLLNICK and B. SALTIN, Distribution of LDH in human skeletal muscle *Scand J clin. Lab Invest* 1974 33 307 31

MONOD, H nd J SCHERRER, Capacité d trava l statique d un groupe musculaire synergic chez *Soc Biol.* 1957 151 1358–1361

ROHMERT W Determination for the recovery pause for static work of man. *Int Z. angew Ph* 18. 123–164

SJÖDIN B., Lactate dehydrogenase in human skelet l muscle. *Acta physiol. scand* 1976. Supp

STEPHENS, J and A TAYLOR, F tigue of mainta ned luntary muscle contraction in man J (Lond.) 1972, 220 1 18

THORSTENSSON A. and J KARLSSON, F tiguabil ty d f bre composition of h man skeletal mu *physiol. scand.* 1976, 98 318–322.

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Effect of Changes in Blood Volume Distribution on Circulatory Variables and Plasma Renin Activity in Man

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Abstract

ård, S J Castenfors and L. E. Lindblad *Effects of changes in blood volume distribution on circulatory variables and plasma renin activity in man.* Acta physiol. scand. 1977 9 237–245

ealthy subjects the pressure around the lower body was changed to 40 mmHg above (LBPP) and (LBNP) atmospheric, thereby altering the amount of blood pooled in the lower body. Heart rate, terial blood pressure, central venous pressure, cardiac output (dye dilution technique) and forearm flow (venous occlusion plethysmography) were measured. Plasma renin activity was determined radioimmunological method. 6 subjects maintained relative circulatory steady state during LBNP caused significant decreases in central venous pressure (CVP), stroke volume and cardiac t ($\dot{Q}$) with an unchanged mean arterial pressure (MAP). Heart rate (HR) increased significantly lated total peripheral vascular resistance (TPVR) and regional vascular resistance (RVR) in the rm were significantly increased when measured 5–9 min after the onset of LBNP whereas plasma activity (PRA) showed definite increase only after 19 min of LBNP stimulation. No correlation was d between the changes in PRA and TPVR or RVR. Increasing the pressure around the lower body P) resulted in slight but significant increase in MAP as well as significant but transient increase in No significant changes were found in HR, $\dot{Q}$ or TPVR. In the forearm decrease in RVR was ustrated. PRA was not significantly changed. The results demonstrate that peripheral vascular tance and PRA are both influenced by changes in blood volume distribution, possibly elicited via thoracic receptors sensitive to changes in central blood volume and/or CVP. The results also est that PRA does not play any significant part in the vasoconstriction during LBNP stimulation.

words: Blood volume, pressor receptors, cardiac output, heart rate, vascular resistance, plasma renin

cording to the classical concept, changes in the constrictor tone of the resistance vessels mediated via baroreceptors in the arterial high pressure circulation (Heymans and Neil 58).

However it has been observed that even a marked increase, of the order of 40 mmHg the transmural pressure of the carotid sinus, simulating a considerable increase in MAP sults in only small changes in RVR (Bevegård and Shepherd 1966, Bevegård, Castenfors d Lindblad, to be published).

On the other hand, during peripheral pooling of blood, causing decreased venous return, ere is marked vasoconstriction, at least in the forearm, before or even in the absence of

BURKE, R E. and R. EDGERTON, Motor units properties and selective involvement in movement. *Exer and sport sciences reviews* 1975 3

CLOSE, R. I Dynamic properties of mammalian skeletal muscles. *Physiol Rev* 1972. 5

ESSÉN, B., E. JANSSON, J HENRIKSSON, A. W TAYLOR, and B. SALTIN, Metabolic characteristics of fibre types in human skeletal muscle. *Acta physiol scand* 1975 95 153–165.

ESSÉN, B. and T HÄGGMARK, Lactate concentration in type I and type II muscle fibres during muscle contraction in man *Acta physiol. scand* 1975 95 344–346.

GOLLNICK, P D J KARLSSON, K PIEHL and B SALTIN, Selective glycogen depletion in skeletal muscle fibres of man following sustained contractions. *J Physiol* (Lond.) 1974 a. 241 59–67.

GOLLNICK, P D., R B ARMSTRONG, C. W SAUBERT K PIEHL and B. SALTIN, Enzyme activity and fibre composition in skeletal muscle of untrained and trained men *J appl Physiol* 1972. 33 312–319

GOLLNICK, P D., B. SJÖDIN, J KARLSSON, E. JANSSON and B. SALTIN, Human soleus muscle: A comparison of fiber composition and enzyme activities with other leg muscles. *Pflügers Arch ges. Physiol* 1974 *348* 247 255

GYDIKOV A., Pattern of discharge of different types of alpha motoneurons and motor units during voluntary and reflex activity under normal physiological conditions. Fifth Intern Congress of Biomechanics, Jyväskylä, Finland 1975

HULTÉN, B A THORSTENSSON B. SJÖDIN and J KARLSSON. Relationship between isometric endurance and fibre types in h man leg muscles. *Acta physiol scand* 1975. 93 135–138

HUMPHREYS, P W and A. R. LIND, The blood flow through active and inactive muscles of the forearm during sustained handgrip contractions. *J Physiol* (Lond.) 1963. *166* 120–135

JORFELDT L., S. FORSBERG, A. JUHLIN DANNFELT and J KARLSSON, Relationship between lactate formation in skeletal muscle nd release t the venous blood during bicycle exercise. Proc. of The Intern Un of Physiological Sciences, New Delhi, 1974

KARLSSON, J and B. SALTIN, Lactate, ATP and CP in working muscles during exhaustive exercise in man *J ppl Physiol* 1970 29 598–602.

KARLSSON, J Lactate nd phosphagen concentrations in working muscle of man. *Acta physiol. scand* 1971 Suppl. 358.

KARLSSON J and B. OLLANDER Muscle metabolites with exhaust ve static e ercise of different duration *A ta physiol scand* 1972 86 309–314

KARLSSON, J C. FUNDERBURK, B ESSÉN nd A. R. LIND, Constituents of human muscle in isometric fatigue *J appl. Physiol* 1975 38 208–211

KARLSSON J K. FRITH B SJÖDIN P D GOLLNICK and B. SALTIN, Distribution of LDH isozymes in h man skeletal muscle. *Scand. J clin Lab. Invest* 1974 33 307 312.

MONOD, H and J SCHERRER, Capacité de travail statique d'un groupe musculaire synergic chez l'Homme *Soc Biol* 1957 *151* 1358–1361

ROHMERT W Determination for the reco ery pause for static work of man. *Int Z. angew. Physiol* 1960 *18*. 123–164

SJÖDIN, B Lactate dehydrogenase in human skeletal muscle. *Act physiol. scand* 1976 Suppl 436.

STEPHENS, J and A TAYLOR, Fatigue of maintained oluntary muscle contraction in man. *J Physiol* (Lond.) 1972. *220* 1 18

THORSTENSSON A. and J KARLSSON, Fatiguabil ty and f bre composition of huma keletal muscle *Acta physiol scand.* 1976. 98 318–322.

...al baroreceptors ...ere stimulated by neck suction. The method for and findings during carotid ...on are presented in ... separate publication (Bevegård et al. 1977).

Methods

...*lar pressure* were measured ...th strain-gauge transducers (Statham P 23 and Statham PBB 30) ...rded on an ultraviolet recorder (ABEM Ultralette). The reference level was the midthoracic level ...upine position. Mean arterial pressure was obtained by electrical integration.

...*c output* (Q) ...as determined by dye dilution technique, using Indocyanine green as indicator ...e cardiodensitometer).

...rate (HR) was monitored continuously by ... heart rate meter (Siven Electromedicine EQ 1).

...*peripheral vascular resistance* ($TPVR$) was calculated as the mean blood pressure minus the central ...pressure divided by the cardiac output and expressed in arbitrary units.

...*rm blood flow* (FBF) ...as measured by venous occlusion plethysmography. The plethysmograph ...d of an air-filled rubber cuff (Graf and Westersten 1959). Forearm blood flow was calculated as the ... of three inflow curves.

...*nal vascular resistance* (RVR) was calculated as the mean arterial pressure divided by forearm blood ...d expressed in arbitrary units.

...*ma renin activity* (PRA) ...as determined with ... radioimmunological technique (NEN commercial kit) ...e ... method published by Haber et al. (1969). No modification of the commercial kit was introduced. ...mples were kept cold and centrifuged within 1 h and the plasma was kept frozen at -20 C until ... All renin values on the same subject were determined in the same incubation and referred to the ...standard curve, to minimize the error of the method. Plasma renin activity was expressed in ...$^{-1}$ h^{-1}. Under these conditions the error of the method is ± 0.1 ng ml^{-1} h^{-1} when measuring ... values in the normal range (0.4–1.6 ng ml^{-1} h^{-1}).

...*tical methods*. Conventional methods were used in the statistical analysis of the data. The dif...s between circulatory data obtained during different blood volume distribution for each subject ...analyzed with Students 't'-test for paired observations. The following probability (P) levels of sig...ce were used:

...001 (xxx), p ... 0.01 (xx), p ... 0.05 (x). P values ... 0.05 are considered significant.

Results

...*odynamic effects of lower body negative pressure* ($LBNP$) -40 *mmHg*

...o of the eight subjects LBNP caused ... progressive fall in blood pressure, resulting in ...ending syncope and necessitating an ...brupt termination of the stimulus. These two ...ects have been excluded from the statistical analysis.

...x subjects maintained a relative circulatory steady state during LBNP: thus only minor ...erences ...ere found between the values obtained during the 5th to 9th min compared ...h the 15th to 19th min of LBNP. The mean of the measurements during the two periods ...presented (Table I). LBNP induced ... significant increase in heart rate and a significant ...rease in pulse pressure, while mean arterial pressure was essentially unchanged. Central ...ous pressure, stroke volume and cardiac output decreased significantly. Total peripheral ...scular resistance increased significantly. Forearm blood flow decreased and regional ...scular resistance increased significantly (Fig. 2).

...*modynamic effects of lower body positive pressure* ($LBPP$) ... 40 *mmHg*

...BPP induced a significant but transient increase in central ...enous pressure. Thus, during ...BPP a significant elevation of central ...enous pressure compared with the control period

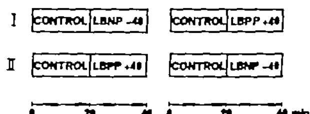


Fig. 1 General experimental design Cases 3 5 and 7 were examined according to scheme I cases 2, 4, 6 and 8 according to scheme II The heavy lines indicate the time hemodynamic data were collected

any detectable change in arterial blood pressure, pulse pressure, dp/dt or HR (Zoller et al 1972, Johnson *et al* 1974) suggesting that the vasoconstriction is mediated via receptors the low pressure side of the circulation

A temporary increase of venous return and CVP elicited by passive lifting of the l results in a dilatation of the resistance vessels of the forearm that does not correlate with magnitude of the increase in arterial pressure and HR suggesting that the vasodilatation mediated via low pressure baroreceptors (Roddie, Shepherd and Whelan 1957).

Data thus support the hypothesis that intrathoracic receptors exert important regula effects on the sympathetic tone of resistance vessels. Recent studies suggest that such rec tors also mediate regulatory effects of the release of renin from the kidney (for rd Davies and Freeman 1976)

The aim of the present investigation was to study central and peripheral circulation PRA during changes in the distribution of blood volume, with the intention of influen mainly receptors in the low pressure system.

Material

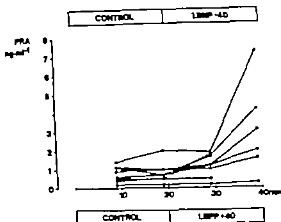
8 healthy male blood donors participated in the study In keeping with institutional policy the inv tion pla was submitted to a approved by the Etical Committee of Södersjukhuset. The observers inf rmed bout the nature and purpose of the nvestigation. Mea values ±S.D of age, weight and h were 31 6±8.8 years, 73.0±7 7 kg nd 1 79±0.08 m.

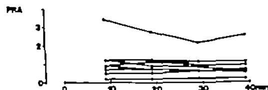
Procedure

The left brachial artery was catheterized percutaneously using a soft teflon catheter (d. 1 40 1 mm). The central venous pressure was bt ined by percutaneous introduction of simila teflon ca via an ntecubital vein. The position of the catheter tip was checked by recording respiratory ch typical for intrathoracic recordings.

The lower body (below the iliac crest) was enclosed in an air tight box. W th the aid of high ca electric pump, the pressure in the box could be cha ged rapidly to 40 mmHg above or below atmosp This procedure will be referred to as lower body negative pressure (LBNP −40) and lo er body po pressure (LBPP +40) respect vely The temperature the box could be mainta ned essentially unch during LBNP −40 and during periods w th tmospheric pressure in the box. During prolonged l +40, however measurement in 2 subjects revealed sl ght gradual ncrease in bo temperature due t efflux from the pump (approximately 4–5°) dur g the timulation period. The measurements were with the subjects lying supine in room with a temperature of bout 22°C.

The experimental design is presented in Fig. 1 Every ubject was examined duri g four periods, lasti g 20 min and differing only with respect to the pressure surroundi g the lower body The seq of LBNP −40 and LBPP +40 was alternated i every second case ccording to Fig. 1 Measurem presented in this study were collected in the period 5–9 min nd 15–19 min. B the 10th and 15th





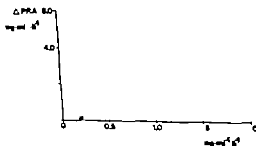
Effect on PRA of LBNP and LBPP. The dotted lines refer to the tw... with impending syncope. The open ... in the picture below refer to sub... who were investigated according to ... I in Fig. 1. In these subjects the ... values may be influenced by the ... ing LBNP procedure.

the two subjects with impending syncope, the plasma renin activity pattern during the d studied was similar to that in the six subjects who maintained a relative circulatory ly state (Fig. 3).

e increase in plasma renin activity during the LBNP period showed a positive correla- to the initial plasma renin activity value (r 0.92) during the preceding control period . 4)

Discussion

...er body negative pressure (LBNP) results in sequestration of some of the blood volume he lower part of the body. This pooling arises from an increase in the transmural pressure he affected vessels, mainly resulting in an increase of the amount of blood pooled in the ns. The amount of blood pooled during LBNP –40 has been estimated to be in the order



. 4. Increase in PRA during LBNP as ...ction of the initial PRA value. The in... ...was calculated as the difference between ... value obtained at 15–19 min of LBNP ...d the mean of the two values during ...ntrol conditions.

TABLE I Effect of LBNP −40 (n = 6) and LBPP +40 (n = 8) on some hemodynamic variables. Mean values ±S.E.

	Control	LBNP −40	Diff	Sign. of diff	Control	LBPP +40	Diff	Sign. of diff
Mean arterial pressure	93.5	93.8	+0.3	n.s.	94.6	99.0	+4.4	p [illegible]
(mmHg)	±2.5	±2.9	±0.1		±2.3	±2.6	+1.2	
Pulse pressure	49.8	43.0	+6.8	p<0.05	50.5	51.4	+0.9	[illegible]
(mmHg)	±1.9	±3.0	±1.7		±[illegible]1	±3.2	±[illegible]5	
Central venous pressure	6.5	2.1	−4.3	p<0.01	7.5	8.8	+1.3	n.s.
(mmHg)	±1.0	±1.3	±0.5		±0.7	±1.0	±0.6	
Cardiac output	6.4	5.[illegible]	−1.2	p<0.01	5.9	6.2	+0.3	n.s.
($1\ min^{-1}$)	±0.3	±0.2	±0.3		±0.4	±0.5	±0.3	
Heart rate	71.8	83.7	+11.8	p<0.01	69.3	70.3	+1.0	n.s.
($beats\ min^{-1}$)	±1.7	±3.1	±1.8		±2.6	±2.[illegible]	±1.6	
Stroke volume	89	63	−26	p<0.01	86	89	+3	n.s.
(ml)	±5	±4	±1		±7	±8	±3	
Total peripheral vascular resistance (units)	13.7	17.8	+4.1	p<0.01	15.2	15.9	−0.1	n.s.
	±0.6	±0.7	±0.7		±1.2	±1.1	±0.7	

was found only in the period 5–9 min after the onset of LBPP (+2.1 mmHg, p 0.05). In the period 15–19 min, central venous pressure was essentially restored to control value. Comparing the mean values during LBPP with those during the preceding control period (Table I) showed a slight but significant increase in mean arterial pressure (+4.4 mmHg, $p < 0.05$). No significant change was found with respect to heart rate, pulse pressure, stroke volume, cardiac output or total peripheral vascular resistance. Forearm blood flow increased significantly resulting in a significant decrease in regional vascular resistance (Fig. 2).

Effect of lower body positive pressure (LBPP) and lower body negative pressure (LBNP) on plasma renin activity

LBPP induced no significant changes in plasma renin activity (Fig. 3). After 9 min of LBNP there was only a slight increase in plasma renin activity whereas after 19 min plasma renin activity was increased to approximately three times the control value.

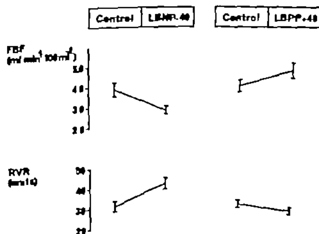
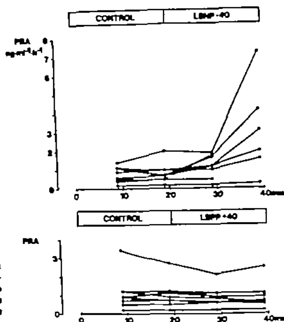


Fig. 2. Effects of LBNP (n = 6) and L[illegible] (= 8) [illegible] peripheral circulation [illegible] values ±S.E. [illegible] given. Asterisk [illegible] significance of deviation. Forearm [illegible] flow (FBF). Regional vascular resist[illegible] in the fore[illegible]



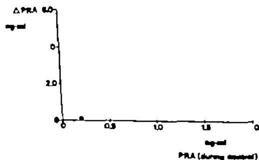
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Discussion

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...4. Increase in PRA during LBNP as ... of the initial PRA value. The ... calculated as the difference between ... values obtained at 15–19 min of LBNP ... the mean of the two values during ... control conditions.

of 0.5–0 6 l (Murray *et al* 1967 Musgrave *et al* 1969) LBNP –40 should therefore be adequate stimulus for studying the effects of a moderate central hypovolemia in intact man

The demonstrated reduction in central venous pressure by approximately 4 mmHg elicited by LBNP –40 and the reduction in cardiac output by 1.2 l min⁻¹ are in accordance with earlier studies (for ref see Wolthuis, Bergman and Nicogossian 1974). Mean arterial pressure was essentially unchanged, indicating a substantial active constriction of the resistance vessels, reflected in the significant increase in both total peripheral and regional vascular resistance.

No direct measurements on the redistribution of blood elicited by lower body positive pressure (+40 mmHg) during steady state conditions have been found in the literature. In a study of the central hemodynamic effect in supine position of inflating an antigravity suit covering the legs and the lower abdomen to 80 mmHg, Gray *et al.* (1969) demonstrated only a transient increase in central blood volume, reflected in a short lasting elevation of central venous pressure.

In the present study central venous pressure after an initial rise, showed a similar gradual decrease towards control value However 5–9 min after the onset of LBPP there was still an elevation of 2.1 mmHg ($p < 0.05$), suggesting that the present method of applying counter pressure around the whole lower body may be slightly more effective than the antigravity suit

LBNP and LBPP elicited opposite changes *i.e* a decrease and an increase respectively in forearm blood flow Although a slight increase in mean arterial blood pressure was found during LBPP in this material, the unchanged mean arterial pressure during LBNP suggests that the recorded changes in forearm blood flow cannot simply be attributed to changes in mean arterial pressure, *i.e* perfusion pressure, but also depend on reflexogenic changes in constrictor tone.

Even if the changes in mean arterial pressure are small, it is probable that changes in arterial baroreceptor activity secondary to for example, changes in heart rate, pulse pressure, dp/dt or intrathoracic blood volume, may contribute to the demonstrated changes in regional vascular resistance. However a direct stimulation of the carotid sinus by neck suction (Bevegård *et al* to be published) or manual compression of the carotid artery (Roddie *et al.* 1957), simulating a considerable increase and decrease respectively in intrasinus pressure results in only small changes in regional resistance in a forearm segment. This suggests that the demonstrated changes in the forearm blood flow are only partially due to changes in the carotid sinus baroreceptor activity

Recent studies indicate that the vasoconstriction in the forearm during LBNP is mediated mainly via intrathoracic receptors sensitive to changes in central venous pressure and central blood volume (Zoller *et al* 1972, Johnson *et al* 1974) It has also been suggested that low pressure baroreceptors are more important than the carotid baroreceptor for the increase in forearm vascular resistance during up-right tilt (Abboud *et al* 1975) Murray *et al* (1959) showed that LBNP caused only minor and inconsistent changes in intrathoracic pressure as assessed from intraesophageal pressure, whereas central venous pressure showed a consistent decrease. This indicates that LBNP decreases the transmural pressure of the right atrium or central veins which may be the stimulus that elicits the vasoconstriction in resistance vessels

ssive leg elevation to drain blood into the thorax, a stimulus that is probably
ature to the LBPP used in this study. Roddie *et al.* (1957) implicated that low
oreceptors mediate a reflex vasodilatation in the forearm when the intrathoracic
me increases. The present finding of an increase in forearm blood flow and a
regional vascular resistance might partially be explained by such a mechanism.
equence of changes during prolonged LBPP stimulus suggests, however, that the
forearm blood flow during LBPP in this study cannot depend exclusively on this
c mechanism. If the vasodilatation depended solely on low pressure barorecep-
radual decrease in central venous pressure during the continuous LBPP should
paralleled by gradual decrease in forearm blood flow, whereas a slight increase
t found. The explanation might be an increase in the skin blood flow due to in-
ting (Crossley *et al.* 1966, Detry *et al.* 1972), as the temperature around the lower
eased slightly during the stimulation period. The change in forearm blood flow
BPP probably reflects the net result of interacting thermo- and baroreceptors.
rease in plasma renin activity during LBNP agrees with the findings by Fasola and
972) and Mancia, Romero and Shepherd (1976). The release of renin from the
s influenced by complex sympathetic and intrarenal mechanisms (Davis *et al.* 1976).
ase in plasma renin activity during LBNP in this study might be elicited by changes
mpathetic stimulation of the juxtaglomerulus apparatus and/or changes in renal
striction influencing intrarenal mechanisms.

calization of circulatory receptors affecting renin release is controversial. Some
gest that the carotid baroreceptor (Hodge, Lowe and Vane 1966, Bunag, Page and
on 1966) may influence renin release, but reports in the literature are contradictory—
et al. (1974) recently demonstrated that pressure changes in the carotid sinus do
n to have any demonstrable effect on plasma renin activity in dogs. Brennan *et al.*
ave demonstrated that receptors on the venous side of the circulation, possibly in
t atrium, are involved in the regulation of renin release and Mancia *et al.* (1975)
showed that vagal afferents from cardiopulmonary receptors influence the release
n. It seems probable that the LBNP stimulus used in the present study affects recep-
the venous side distinctly more than those on the high pressure side, as mean arterial
re was unchanged. This supports the hypothesis that the release of renin is elicited by
oracic receptors.

gnificant increase in plasma renin activity was demonstrated only at the end of the
period (*Fig.* 3), indicating that the rapid increase in total peripheral vascular re-
ce and in regional resistance was not related to the vasoconstrictive effect of angio-
II, which is known to be potent vasoconstrictor. Also, the finding of large inter
dual variations in the increase in plasma renin activity during LBNP (Fig. 3), not
d to the changes in total peripheral vascular resistance or regional vascular resistance,
sts that angiotensin II contributes little to the increased vascular resistance during
P. This is in accordance with the finding of Kala, Fyhrquist and Eisalo (1974), suggest
hat angiotensin II does not play a significant role in the maintenance of blood pressure
ediately after the change of posture from supine to upright. The two subjects with
nding syncope showed much the same plasma renin activity response as the subjects

with a relative stable circulation, further supporting the hypothesis that an angiotensin mediated vasoconstriction is of little importance for short-term adjustment of peripheral vascular resistance.

Angiotensin II is also a stimulus to an increased aldosteron secretion and in this way participates in the regulation of the blood volume. A nervous mechanism mediated through the sympathetic renal nerve influences plasma renin activity during blood volume change, but also intrarenal mechanisms may contribute (Castenfors, Johnson and Orö 1973). The main determinant of the large individual variation in plasma renin activity during LBNP in this study was the initial plasma renin activity value under basal conditions (Fig. 4). Sodium balance and intravascular volume are important for the regulation of the renin response to acute postural changes (Tuck, Dluhy and Williams 1975). All subjects were on free salt and water intake and thus interindividual variations in sodium balance may contribute to the great variations in plasma renin activity response to LBNP

References

ABBOUD, F M A L MARK D D HEISTAD, D L ECKBERG and P G SCHMID, Selectivity of autonomic control of the peripheral circulation in man *Trans Amer clin. climat Ass.* 1975. 86. 184-197.

BEVEGÅRD, S B. and J T SHEPHERD, Circulatory effects of stimulating the carotid arterial stretch receptors in man at rest and during exercise. *J clin. Invest* 1966. 45 132 142.

BEVEGÅRD, S. B., J CASTENFORS and L. E. LINDBLAD, Effect of carotid sinus stimulation on cardiac output and peripheral vascular resistance during changes in blood volume distribution in man. To be published.

BEVEGÅRD, S. B., J CASTENFORS, L. E. LINDBLAD and J TRANESJÖ, Blood pressure and heart rate regulating capacity of the carotid sinus during changes in blood volume distribution in man *Acta physiol scand* 1977 99 300-312.

BRENNAN, L. A. JR, R L. MALVIN K. E. JOCHIM and D E. ROBERTS, Influence of right and left atrial receptors on plasma concentrations of ADH and renin *Amer J Physiol.* 1971 221 273-278.

BRENNAN L. A., A L HENNINGER J E. JOCHIM and R. L. MALVIN, Relationship between carotid sinus pressure and plasma renin level *Amer J Physiol.* 1974. 227 295-299

BUANG, R. D I H PAGE and J W MCCUBBIN, Neural stimulation of release of renin. *Circulat Res.* 1966. 19 851-858.

CASTENFORS, J H JOHNSSON and L. ORÖ, Effect of Alprenolol on blood pressure and plasma renin activity in hypertensive patients. *Acta med. scand* 1973. 193 189 195.

CROSSLEY R. J A D M GREENFIELD, G C. PLASSARAS and D STEPHENS, The interrelation of thermoregulatory and baroreceptor reflexes in the control of the blood vessels in the human forearm. *J Physiol.* (Lond.) 1966. 183 628-636.

DAVIS, J O and R. H FREEMAN, Mechanisms regulating renin release. *Physiol. Rev* 1976. 56 1-56.

DETRY J M R G L BRENGELMANN, L. B. ROWELL and C. WYSS, Skin and muscle components of forearm blood flow in directly heated resting man *J appl Physiol* 197_ 32 506-511

FASOLA, A. F and B. L. MARTZ, Peripheral venous renin activity during 70° tilt and lower body negative pressure. *Aerospace Med* 1972. 43 713-715

GRAF K. and A WESTERSTEN, Untersuchungen über Eigenschaften und Verwendungsmöglichkeiten eines flexiblen Extremitätenplethysmografen *Acta physiol scand.* 1959 46 1 18.

GRAY III S., J A. SHAVER, F W KROETZ and J J LEONARD, Acute and prolonged effects of G-suit inflation on cardiovascular dynamics. *Aerospace Med.* 1969 40 40-43

HABER, E., T KOERNER, L. B. PAGE, B. KLIMAN and A. PURNODE, Application of radioimmunoassay for angiotensin I to the physiologic measurements of plasma renin activity in normal human subjects *J clin. Endocr* 1969 29 1349-1355

HEYMANS, C. and E. NEIL, *Reflexogenic areas of the cardiovascular system* ... and A. C ... London.

[illegible] L., R. D. Lowe and J. R. V[illegible], Increased angiotensin formation in response to carotid [illegible] in the dog. *Nature* (Lond) 1966. *211* 491–492.

[illegible] J. M., L. B. Rowell, M. Niederberger and M. M. Eisman, Human splanchnic and forearm [illegible] responses to reductions of right atrial and aortic pressures. *Circulat. Res.* 1974. *34* [illegible]

[illegible] F. Fyhrquist and A. Eisalo, Effect of short-term upright posture on plasma angiotensin II [illegible] *Scand. J. clin. Lab. Invest.* 1974. *33*, 87–94.

[illegible] G., J. C. Romero and J. T. Shepherd, Continuous inhibition of renin release in dogs by [illegible] [illegible] receptors in the cardiopulmonary region. *Circulat. Res.* 1975. *36*, 579–535.

[illegible] R. H. J. Kroo, L. D. Carlson and J. A. Bowers, Cumulative effects of [illegible] and body negative pressure. *Aerospace Med.* 1967. *38*, 243–[illegible]47

[illegible] R. H., L. J. Thompson, J. A. Bowers and C. D. Albright, Hemodynamic effects of graded [illegible] and vasodepressor syncope induced by lower body negative pressure. *Amer. Heart J.* 196[illegible] [illegible]–431

[illegible] F. S., F. W. Zechman and R. C. Mapes, Changes in total leg volume during lower body [illegible] pressure. *Aerospace Med.* 1969. *40*, 602–606.

[illegible] J. C. and J. T. Shepherd, The effects of carotid artery compression in man [illegible] with special reference [illegible] in vascular resistance in the limbs. *J. Physiol.* (Lond.) 1957. *139* 377–384.

[illegible] J. C., J. T. Shepherd and R. F. Whelan, Reflex changes in vasoconstrictor tone in human [illegible] muscle in response to stimulation of receptors in [illegible] low-pressure area of the intrathoracic [illegible] bed. *J. Physiol.* (Lond.) 1957. *139* 369–376.

[illegible] L., R. G. Dluhy and G. H. Williams, Sequential responses of the renin–angiotensin–aldo[illegible] [illegible] to acute postural change: effect of dietary sodium. *J. Lab. clin. Med.* 1975. *86*, 754–763.

[illegible] R. A., S. A. Bergman and A. E. Nicogossian, Physiological effects of locally applied reduced [illegible] in man. *Physiol. Rev.* 1974. *54* 566–595.

[illegible] R. P., A. L. Mark, F. M. Abboud, P. G. Schmid and D. D. Heistad, The role of low pressure [illegible] receptors in reflex vasoconstrictor response in man. *J. [illegible]. Invest.* 1972. *51* 2967–2972.

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Effects of Carbachol and Calcium on the Cyclic Guanosine-3 ,5 -Monophosphate (Cyclic GMP) Metabolism in Intestinal Smooth Muscle

By

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Abstract

NILSSON K B and R. G G ANDERSSON *Effects of carbachol and calcium on the cyclic guanosine-3 ,5 -monophosphate (cyclic GMP) metabolism in intestinal smooth musc[le]* Acta physiol scand. 1977 99 246–253

In a submaximal concentration carbachol contracted the rabbit colon muscle and increased the cyclic GMP level. The cyclic AMP level was reduced. In a Ca^{++}-depleted muscle carbachol reduced the cyclic GMP level while the effect on the cyclic AMP content of the muscle was unchanged. Carbachol had no effect on the guanylate cyclase activity of the "plasma membrane fraction (the 35–45% fraction) in the homogenate and the microsomal fractions Ca had no effect on the gua ylate activity hile it stimulat[ed] the enzyme in a soluble fraction. In the "plasma membrane fraction cyclic GMP released Ca^{++} from the preloaded fraction and inhibited the Ca^{++} accumulation These effects were not found in the vesicul[ar] microsomal fraction (the 35% fraction). In both fractions, however cyclic GMP counteracted the stimulat[ing] effect of cyclic AMP These results indicate that cyclic AMP and cyclic GMP may have antagonistic roles on the Ca^{++} metabolism in the colon muscle It is suggested that cyclic GMP may act as some kind of positive feedback mechanism which may have mod lating effect on the release of Ca^{++} from one pool to another in rabbit colon.

There is accumulating evidence that the relaxing action induced by β-adrenoceptor agonist[s] is mediated by cyclic AMP (Andersson 1972, Bär 1974) Contracting drugs, on the oth[er] hand, have variable effects on the cyclic AMP levels in different smooth muscles (Anderss[on] 1972, Namm and Leader 1976). In some smooth muscle preparations, however the contrac[-] tion seems to be associated with an increase in the cyclic GMP concentration (Goldbe[rg] *et al* 1975) Thus, it was early found that cholinergic agonists increased the cyclic GM[P] level in guinea pig ileum (Lee *et al* 1972). Subsequently Schultz *et al* (1973) found th[at] several contracting drugs increased the cyclic GMP level in smooth muscles and they fou[nd] this effect to be Ca^{++}-dependent

To elucidate the role of cyclic GMP in rabbit colon muscle, we have studied the effect [of] carbachol and calcium on the guanylate cyclase activity in this preparation. This work al[so] deals with the effect of cyclic GMP on the calcium metabolism in a microsomal fractio[n] ... rabbit colon

Methods

...nal smooth muscle was prepared from rabbit colon. The rabbit was stunned by blow to the neck ...sanguinated. The colon muscle was dissected free of mucosa and pieces of the muscle were attached ...ically to plastic holders and immersed in Krebs–Henseleit bicarbonate buffer at +37°C as described ...dholm and Mohme-Lundholm (1966). The tension was recorded by FT03 transducers on Grass ...raph. In some expts. the muscle was incubated in Ca^{++}-free Krebs–Henseleit bicarbonate buffer. I... expts. the muscle preparations were treated several times with 2 10^{-4} M EGTA to reduce the Ca^{++} ...nt of the muscle before the addition of the drug. At different times after the addition of carbachol ...e muscle they were frozen in Freon 12 and solid CO_2. The colon muscles were homogenized in 5% ...ic acid and the cyclic nucleotides were separated by chromatography on columns of AG-1 X8 ...d with formic acid and lyophilized. The cyclic GMP content was estimated according to Steiner *et al.* ...) and the cyclic AMP according to Gilman (1970). The guanylate cyclase activity was determined by ... reaction buffer containing 40 mM Tris-HCl (pH 7.4), 2 mM GTP 10 mM theophylline, 0.1 mM ...l_2 and other additions as indicated in the figure legends. The reaction was initiated by the addition of ... 100 µg Triton-dispersed proteins from different colon muscle fractions (see below). After incubation ...37°C for 5 min, the reaction was terminated by the addition of 5 PCA. The formed cyclic GMP ...separated and analysed as described above.

...e Ca^{++} accumulating microsomal subfractions were isolated according to Carsten (1969) with some ...ifications (Nilsson *et al.* 1976). The crude microsomal fraction isolated between 17 300–40 000 g ...suspended in 0.08 M NaCl and 0.005 M Na_2-oxalate (pH 7.0), and placed on discontinuous sucrose ...ient containing 35%, 45 and 55 sucrose. The gradient was centrifuged for 120 min at 50 000 g ...protein layers were obtained at 35%, 35–45% and the 45–55% sucrose respectively. In this study we ... used the fractions isolated at 35% and 35–45 sucrose.

...he supernatant from the 40 000 g centrifugation was centrifuged at 100 000 g for 60 min and the ...sulting supernatant was used in the guanylate cyclase assay. The homogenate and the soluble fraction used ...e assay were dispersed in Triton X 100 to final Triton concentration of 1% and kept at +4°C for ... before use.

...he Ca^{++} accumulating studies were performed in an incubation medium containing 0.11 M KCl, 0.01 ...NaCl, 0.02 M histidine buffer (pH 7.2), 0.35 mM $MgCl_2$, 0.35 mM ATP and 9 10^{-6} M Ca^{++} (adjusted ...atomic absorption spectrometer) and containing 0.008 µCi ^{45}Ca. The tests were performed at +37°C, ...ted by the addition of 100–150 µg protein to the medium and interrupted after fixed time intervals by ...filtration through Millipore filter (0.45 µm). The accumulation of Ca^{++} to the microsomes was deter... ...ed by liquid scintillation technique and was calculated from the activity in the unfiltrated and the filtrated ...tion mixtures. Correction was made for Ca bound to the filter. The protein content was estimated accord... ...to Lowry *et al.* (1951).

...tatistical analysis. Mean values are given ± standard error of the mean. The control and the treated ...ples in every separate experiment were obtained from the same animal or microsomal preparation. The ...ificance of the effect was calculated by Student's t-test from the difference between these paired samples.

Results

...e effect of carbachol on the cyclic nucleotide metabolism in the intact colon muscle

...rbachol in a concentration of 4.5 10^{-4} M elicited a submaximal contraction of rabbit ...lon muscle. The contraction was associated with a 15% reduction ($p < 0.05$) of the muscle ...vel of cyclic AMP after 10 s confirming earlier results on this preparation (Andersson ...973). This effect was still present after 60 and 120 s. The muscle content of cyclic GMP was ...creased by carbachol treatment, an increase of about 50% (p 0.05) from a basal value ...(42.5 ± 6.8 pmol/g wet weight was found 10 after the addition of the drug, an effect which ...as also found after 60 and 120 s (Fig. 1). In calcium-depleted muscle preparations the ...arbachol induced effect on the cyclic GMP content was reversed (Fig. 1) while the effect ...n cyclic AMP was still present.

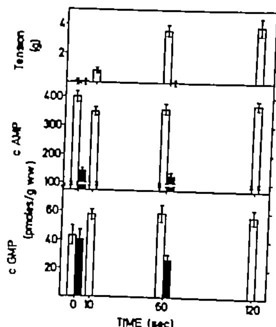


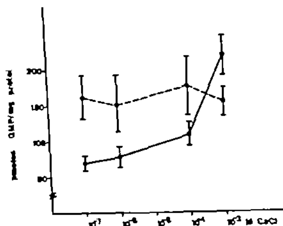
Fig. 1 Influence of carbachol (4.5 10⁻ M) on tension, the cyclic AMP and the cyclic GMP con of normal (open bars) and Ca -poor (filled b rabbit colon muscle. Mean ± S.E. (n 7)

Effects of carbachol and calcium on the cyclic GMP formation of subcellular fractions of rabbit colon

From rabbit intestinal smooth muscle two submicrosomal fractions, the 35 % and the 35–45% fraction had been isolated The biochemical and the morphological investigations indicated that the 35% fraction was of vesicular nature and originated from the plasma membrane as well as the sarcoplasmic reticulum. The 35–45 fraction was a nonvesicular membrane fraction probably mainly consisting of fragments of the plasma membrane (Andersson and Nilsson 1976)

In a previous study (Andersson *et al* 1975) we have found that carbachol reduced the concentration of cyclic AMP in the colon as well as the activity of adenylate cyclase in 35–45 microsomal fraction. Since carbachol increased the cyclic GMP content of the colon muscle it was of interest to investigate its effect on the guanylate cyclase activity. In the 35–45% microsomal fraction 4 8 ± 1 0 pmol cyclic GMP was formed per mg protein. Carbachol did not significantly change the guanylate cyclase in this fraction and the activity was 4 3 ± 0.5 pmol/mg protein/5 min in the presence of the cholinergic agonist.

Since carbachol caused an increase of the cyclic GMP level which was found to be Ca dependent (Fig. 1), we have therefore studied the effect of Ca on the guanylate cyclase activity in different subfractions from colon As indicated in Fig. 2 Ca ions in the concentration range 5 10 to 5 10^{-4} M had no effect on the guanylate cyclase activity of the Triton-dispersed homogenate. The guanylate cyclase activity differed in the two microsomal fractions most of the activity was found in the 35 fraction, 26.3 ± 51 1 pmol/mg protein, while the activity of the 35–45% fraction was only about a quarter of that of the 35 fraction (Table 1). Calcium in the concentration range 10 –10^{-4} M was examined and was found to have no significant effect on the guanylate cyclase activity of the microsomal fractions (Table I).



2 Effect of $CaCl_2$ (5 10^{-} 5 10^{-4} on the formation of cyclic GMP in Ca-dependent homogenate (—) and soluble fraction (—). Mean ± S.E. 3-7)

Most of the guanylate cyclase activity of the colon homogenate was found in the 100 000 g pernatant fraction. Lower concentrations of Ca^{++} (5 10^{-} to 5 10^{-4} M) did not influence the cyclic GMP formation of the fraction (Fig. 2). Higher concentrations of Ca^{++} 10^{-4} and 5 10^{-4} M) significantly increased the guanylate cyclase activity in comparison e activity in the absence of added Ca^{++} was estimated to be 68.3 ± 6.9 pmol/mg protein.

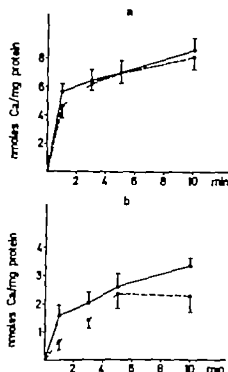
he effect of cyclic GMP on the accumulation and the release of Ca the microsomal fractions

The accumulation of Ca^{++} to the microsomal fractions of 35% and 35–45% sucrose was timulated by cyclic AMP (10^{-4} M) (Nilsson 1973). It was noticed that the addition of yclic GMP to the corresponding fractions had a tendency to reduce the Ca^{++}-binding capacity (Fig. 3). In the 35% sucrose fraction cyclic GMP (10^{-4} M) initially reduced the Ca^{++} binding but did not influence the maximal binding capacity of the fraction (Fig. 3 a). Cyclic GMP treatment of the 35–45% fraction tended to reduce the Ca^{++}-binding within the space of 1 minute and this tendency was observed even after 10 min of incubation with the nucleotide (p 0.1) (Fig. 3 b).

As the cyclic GMP level of the intact muscle was increased during the contraction it was of interest to investigate whether cyclic GMP influenced any calcium-releasing mechanism

TABLE I. The effect of different concentrations of Ca^{++} on the cyclic GMP formation in microsomal fractions isolated from rabbit colon. The incubation time was 5 minutes. Mean ± S.E. — 6–7

Experimental conditions	Cyclic GMP formation (pmol/mg protein, 5 min)	
	35	35–45
Basal value	26 3 ± 5.1	4 0 ± 1.5
$CaCl_2$ (1 10^{-7} M)	31 8 ± 4.2	5 0 ± 0.9
$CaCl_2$ (1 10^{-4} M)	27.0 ± 4 7	6.3 ± 1 4
$CaCl_2$ (1 10^{-4} M)	25 6 ± 5.1	5.7 ± 1 0



a

Bound Ca (%)

100 80 60 40 20

2 4 6 8 10 min

b

Bound Ca (%)

100 80 60 40 20

2 4 6 8 10 min

4

Fig. 3. The effect of 10^{-4} M cyclic GMP ( ) on the Ca^{++} binding in a) the 35 % fraction and b) the 35–45 % fraction. The control curve is denoted by — Mean ± S.E. (n = 6–7).

Fig. 4. The effect of 10^{-4} M cyclic GMP (-) on the Ca^{++} release from a) the 35 % fraction and b) the 35–45 % fraction. The fractions were preloaded with ^{45}Ca for 5 min before the addition of cyclic GMP. Mean ± S.E. (n = 6–9). The effect of cyclic GMP on the Ca^{++} release was significantly separated from the control curve (—) (Fig. 4 b). * = $p < 0.05$, ** = $p < 0.01$

in the colon. The addition of cyclic GMP to the microsomes preloaded by Ca^{++} for 5 min caused a relase of Ca^{++} from the 35–45 % sucrose fraction (Fig. 4 b). Initially the release was very rapid and was probably significant already after 1 min treatment compared to the control expts. A further release was observed after 3 to 10 min incubation. In the 35 % sucrose fraction cyclic GMP (10^{-4} M) had no effect on the Ca^{++} release (Fig. 4 a).

An antagonistic effect of cyclic GMP on the cyclic AMP stimulated Ca accumulation

George *et al* (1973) reported that cyclic GMP added simultaneously with cyclic AMP reduced the Ca^{++}-binding capacity of the cardiac microsomes compared with cyclic AMP alone. This data support the hypothesis that cyclic AMP and cyclic GMP may play opposite roles in the regulation of cardiac contraction. It was of interest to investigate whether there existed a similar relationship in intestinal smooth muscle microsomes. Simultaneous addition of cyclic GMP (1 × 10^{-} M to 1 × 10^{-4} M) and cyclic AMP (1 × 10^{-} M) to colon microsomes was performed and the effects were compared with Ca^{++}-accumulation in the presence of cyclic AMP alone. Already at the concentration ratio of 100 : 1 of cyclic AMP : cyclic GMP a moderate antagonistic effect was observed in the 35 % sucrose fraction (Table II). The reduction of the Ca^{++}-accumulation was about 20–30 %.

I The influence of cyclic GMP on the Ca^{++} accumulating microsomal fractions of rabbit colon muscle incubated in the presence of cyclic AMP. The fractions having been incubated for 5 minutes in the presence of these agents. Mean ± S.E. = 6. Significance degree = p 0.05

...ental conditions		Reduction of the Ca^{++} accumulation (%)	
...AMP	Cyclic GMP	35	35–45
	(M)		
	10^{-7}	19 ± 3	30 ± 17
	5 10^{-7}	12 ± 4	30 ± 13
	1 10^{-6}	19 ± 6	50 ± 18
	5 10^{-6}	22 ± 9	50 ± 18
	1 10^{-5}	32 ± 11	55 ± 19

the fraction 35–45 %, sucrose the stimulating effect of cyclic AMP was significantly the ratio of 20 1 (Table II). The inhibition was optimal at the ratio 10 1 and estimated to be about 50 %.

Discussion

enhancement of the cyclic GMP level of the intact colon muscle during contraction by achol was Ca^{++}-dependent. This is in accordance with the findings on rat ductus defe (Schultz *et al.* 1973), where several agents that increased the cyclic GMP level have a $^{+}$ requirement for their action. Cholinergic agonists increased the cyclic GMP level in er smooth muscles too. Lee *et al.* (1972) have observed this effect in guinea pig ileum and er (1974) reported a similar effect from rabbit gallbladder. Angles d'Auriac and Worcel 75) did not find any increase in the rat uterus after carbachol administration, however ere are thus some observations indicating an elevation of the cyclic GMP level in ooth muscles from the gastrointestinal tract after stimulation with cholinergic drugs.

Our study does not support a hormone-receptor mediated activation of particulate guany-e cyclase, since no effect of carbachol was observed on the guanylate cyclase of the micro-mes. The present results and those of Schultz *et al.* (1973) indicate that the most likely anylate cyclase activating factor is Ca^{++}. The soluble guanylate cyclase from colon muscle is stimulated by high concentrations of Ca^{++}-ions (5 10^{-4} M, Fig. 2). Calcium, in latively high concentrations, increased the soluble guanylate cyclase activity of sea urchin erm (Garbers *et al.* 1975) as well as of rat brain (Olsson *et al.* 1976). The concentrations of a that are required for demonstrating effects on guanylate cyclase are so high that its hysiological importance becomes unclear. It must be concluded, however that the incuba-on conditions used in most *in vivo* studies are not identical with the "milieu interieur" of mooth muscle cells, and the enzymatic activities *in vivo* may therefore have different require-nents of calcium.

The physiological role of cyclic GMP in smooth muscle contraction is unclear. W have suggested that cyclic GMP may promote contraction by acting as modulator on Ca^{++} release in the smooth muscle cell (Andersson *et al.* 1975). Contradictorily cyclic GMP has been suggested as a negative feed-back signal which tends to lower the cytoplasmic Ca^{++}

concentration (Schultz *et al* 1975) The results of the present investigation provide a theoretical basis for cyclic GMP as a modulating factor for Ca release in colon muscle since the nucleotide caused a release of Ca^{++} from one of the microsomal subfractions (Fig. 4). Cyclic GMP also counteracted the stimulating effect of cyclic AMP on the Ca^{++} accumulation in colon microsomes (Table II) which is in conformity with the observations on cardiac microsomes (George *et al* 1973)

The results presented earlier by Schultz *et al* (1973) and Andersson *et al* (1976) along with the findings in this work have led us to the working hypothesis. The interaction of cholinergic agonists with muscarinic receptors on the colon muscle membrane causes a depolarization and an increase of the myoplasmic Ca^{++} concentration. Ca ions may stimulate soluble guanylate cyclase and increase the cyclic GMP level The elevation of cyclic GMP may be a factor of importance for the maintenance of contraction by supporting the Ca^{++}-release from certain subcellular calcium-pools.

It appears likely that cyclic GMP may be a modulating factor in rabbit colon contraction We have to be cautious in our generalizations, since the action of cholinergic agonists are without effect on cyclic GMP levels of uterine smooth muscle Angles d'Auriac and Worcel (1975) Further in indomethacin-treated guinea-pig gallbladder we have found that the contraction induced by cholecystokinin is unaffected although the increase of cyclic GMP has failed to appear (Andersson *et al.* 1976)

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References

AMER, M S., Cyclic guanosine 3,5 monophosphat and gallbladder contraction. *Gastroenterology* 1974 *67* 333–337

ANDERSSON, R Cyclic AMP and calcium ions i mechanical and metabolic responses of smooth muscle. Influence f some hormons and drugs. *A t physiol. scand.* 1972. Suppl. 382. 1 59

ANDERSSON, R. G G Relationship between cyclic AMP phosphodiesterase activity calcium and contraction in intestinal smooth muscle. *Acta physiol. scand* 1973 *87* 348–358

ANDERSSON R., K. NILSSON, J WIKBERG, S JOHANSSON, E. MOHME LUNDHOLM and L. LUNDHOLM, Cyclic nucleotides and the co traction of mooth muscle. I *Advances in Cyclic Nucleotide Research* 1975 *5* 491 518 Ed by Drummond, G I Greengard, P and Robinson, G A., Ra en Press, N Y

ANDERSSON, R. G G and K. B NILSSON, Role of cyclic nucleotides for metabolism and mecha ical activity i smooth muscle. I *The biochemistry f Smooth Muscle* 1976. Ed. by N L. Stephens, University Park Press. In press.

ANDERSSON, K. E., R. G G ANDERSSON, P HEDNER and C. G A. PERSSON, Interrelations between cyclic AMP cyclic GMP and contraction in guinea pig gallbaldder stimulated by cholecyst kinin. *Life Sci* 1976. In press.

ANGLES d AURIAC, G and M WORCEL, Variations in cGMP and cAMP levels in rat uterine smooth muscle induced by carbachol, $PGF_{2\alpha}$ and changes in ionic composition. *Brit J Pharmacol.* 1975 *55* 236p–237p.

BÄR, H. P Cyclic nucleotides nd smooth muscle. I *Advance in Cyclic Nucleotide Research* 1974 *4* 195–237 Ed. by Greengard P and Robinson, G A., Raven Press, N Y

CARSTEN, M E., Role of calcium binding by sarcoplasmic reticulum in the contraction and relaxation of uterine smooth muscle. *J gen. Physiol.* 1969 *53* 414–4 6.

GARBERS, D L., E. L. DYER and J G HARDMAN, Effects of cations on gua ylate cyclase of sea urchin sperm. *J biol. Chem.* 1975 *25* 382 387

W J L. A. White and R. D. Wilkerson, Effects of guanosine 3',5'-monophosphate (cGMP) and adenosine 3',5'-monophosphate (cAMP) on calcium accumulation in rat cardiac microsomes. Fed. Proc. 1973 32, 711.

[illegible], A. G A protein binding assay for adenosine 3',5'-monophosphate. Proc. nat. Acad. Sci. (Wash.) 1970 67 305-312

[illegible], N. D M. K. Haddox, S. E. Nicol, D. B. Glass, C. H. Sanford, F A. Kuehl and R. Estensen, Biological regulation through opposing influences of cyclic GMP and cyclic AMP The Yin Yang Hypothesis. In Advances in Cyclic Nucleotide Research 1975. 5 307–330. Ed. by Drummond, G. I., Greengard, P and Robinson, G. A., Raven Press, N.Y

[illegible], T P J. F Kuo and P Greengard, Role of muscarinic cholinergic receptors in regulation of guanosine 3',5' cyclic monophosphate content in mammalian brain, heart muscle and intestinal smooth muscle. Proc nat. Acad Sci (Wash.) 1972. 69 3287–3291

[illegible]ry O H., N. J Rosebrough, A. L. Farr and R. J Randall, Protein measurement [illegible] the folin phenol reagent. J biol. Chem 1951 193 265–275.

[illegible], L. and E. Mohme-Lundholm, Length at inactivated contractile elements, length tension diagram, active state and tone of vascular smooth muscle. Acta physiol. scand. 1966. 68, 347–359

[illegible], D. H and J P Leader, Occurrence and function of cyclic nucleotides in blood vessels. Blood Vessels 1976 13 24–47

[illegible], K., Ca++-binding and release in smooth muscle sarcoplasmic reticulum in relation to cyclic AMP Acta physiol scand. 1973, Suppl. 396 p. 4

[illegible], K L. DeArv and R. G. G. Andersson, Contraction of glycerol extracted fibres of smooth muscle by acetylcholine and [illegible] in the presence of Ca-binding microsomal fraction from rabbit colon. Acta physiol. scand. 1976 98 407–411

[illegible], D. R., C Kon and R. L. Bruckenbourer, Calcium ion effects on guanylate cyclase of brain. Life Sci 1976, 18, 935–940.

[illegible], G J. G Hardman, K. Schultz, C. E. Baird and E. W Sutherland, The importance of calcium ions for the regulation of guanosine 3',5'-cyclic monophosphate level. Proc. nat Acad. Sci. (Wash.) 1973, 70, 3889–3893.

[illegible], G J. G. Hardman and L. Hurwitz, Cyclic nucleotides and smooth muscle function, In Proc 6th Int Congr Pharmacol 1975 1 203–211 Ed. by Tuomisto and Paasonen, Helsinki.

[illegible], A. L., C. W Parker and D M. Kipnis, Radioimmunoassay for cyclic nucleotides. J biol. Chem. 1972. 247 1106–1113.

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Antilipolytic Effect of Adenosine in Dog Adipose Tissue *in Situ*

By

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Adenosine and related compounds are released from isolated rat fat cells (see Schwa *et al* 1975) and from canine subcutaneous adipose tissue (Fredholm 1976). The rate release from the latter preparation is enhanced by sympathetic nerve stimulation. Adenosi inhibits cyclic AMP accumulation in isolated rat fat cells already in submicromolar c centrations (Fain *et al* 1972, Schwabe *et al* 1975), while lipolysis is inhibited to a considera lower degree in this preparation As discussed by Fain (1973) this apparent contradict might be explained if cyclic AMP levels are raised to supramaximal levels by the concent tion of the lipolytic drugs commonly used in studies of isolated fat cells. In a recent study found that maximal rates of lipolysis in canine subcutaneous adipose tissue induced by ne stimulation are associated with only small (50%) increases in tissue cyclic AMP con (Fredholm *et al* 1976)

In the present study we have therefore investigated the effect of adenosine on lipol induced by sympathetic nerve stimulation A preliminary account of some of these d has been given (Fredholm 1975)

21 fed, female mongrel dogs we ghing 8–16 kg were used. Anesthesia was induced with 30 mg kg and pentobarbital i nd supplemented as necessary during the expt. Subcutaneous adipose tissue was iso (Rosell 1966) and autoperfused. Responses were studied in 30 fat pads weighing 20–42 g. The ani were tracheotomized, artificially ventilated and arterial pO_2, pCO_2 and pH controlled. Heparin (2 mg kg) was given to prevent clott g. Adipose tissue blood flow was measured using a drop counter nerve t the preparation was isolated and stimulated at 4 Hz (2 ms, 12 V). Arterial and venous bl samples were drawn nd glycerol (Laurell d T bbling 1966) was assayed on deproteinized plasma Ad sine was administered by close i.a. infusion For futher experimental details see below Dipyrid (Persantin, Boehringer Ingelheim) was administered i.

Adenosine infused i.a. to give concentrations in the arterial blood flow ranging betw 0 01 and 40 μM had no significant effect on the basal rate of glycerol release. In contr glycerol release evoked by electrical nerve stimulation was inhibited by adenosine in c centrations above 1 μM (Table I)

In order to test the possible significance of endogenous adenosine the well-known ade sine uptake inhibitor dipyridamol (Schrader *et al* 1972) was administered to the dogs. results of these expts. are summarized in Table II While ineffective on basal glycerol rele dipyridamol (0.5 mg/kg i v) inhibited stimulated glycerol release.

The present results demonstrate that exogenous adenosine is antilipolytic in canine s cutaneous adipose tissue *in situ* A 70% inhibition was found by administering adenosin

TABLE I Inhibition of stimulated glycerol release by adenosine. $\bar{x} \pm S_{\bar{x}}$.

Adenosine concentration (μM)	Glycerol release (% of control)
3 (4)	108 ± 6
6–0 15 (6)	101 ± 6
3 (8)	117 ± 14
20	74 (61, 87)
40 (4)	27 ± 10 ($p < 0.001$)

Stimulated glycerol release was calculated by summing the total amount of glycerol released during and following nerve stimulation and subtracting the basal release for the same overall time period.

TABLE II Effect of dipyridamol (Persantin®) on glycerol release from canine subcutaneous adipose tissue $\bar{x} \pm S_{\bar{x}}$

	Before dipyridamol	*After dipyridamol*[a]	Per cent of control
Basal release rate (μmol min⁻¹ 100 g⁻¹)	0 153 ± 0 026 (11)	0.156 ± 0 026 (7)	100 ± 16
Stimulated release (μmol 100 g⁻¹)	21 6 ± 3.4 (11)	13.3 ± 3.6 (7) p 0 01	57 4 ± 10 6 (7) p < 0.01[c]

[a] Dipyridamol was administered i. a. in a dose of 0 5–1 mg/kg before nerve stimulation.
[b] Students t test for means of two samples.
[c] Students t-test for one sample.

was calculated to give 10–40 μM concentration in the blood. Since adenosine is rapidly inactivated in blood and tissue (Schrader *et al.* 1972) the concentration of adenosine achieved in the fat cells is probably considerably lower. In isolated rat fat cells noradrenaline stimulated lipolysis was inhibited by less than 25 per cent at 10 μM adenosine (Hjemdahl and Fredholm 1976). Thus adenosine is a far better inhibitor of lipolysis *in vivo* than *in vitro*. Since adenosine probably acts by inhibiting cyclic AMP formation (Fain 1973, Schwabe *et al.* 1975, Hjemdahl and Fredholm 1976) this may be related to the fact that cyclic AMP increases *in vivo* are far smaller than those commonly studied *in vitro* (Fredholm *et al.* 1977).

The recent finding that adenosine inhibits noradrenaline release from several tissues including canine subcutaneous adipose tissue (Hedqvist and Fredholm 1976) could also partially explain the potent antilipolytic effect of adenosine under the present experimental conditions.

Adenosine (and related compounds) is found in the venous effluent following nerve stimulation (Fredholm 1976). It can be calculated that the maximal concentrations achieved are in the μmolar range (Fredholm *et al.* 1977). Since the present results demonstrate that adenosine is antilipolytic under these conditions a physiological role must be considered. Therefore the finding that dipyridamol, a well-known inhibitor of adenosine inactivation (Schrader *et al.* 1977), decreased the lipolytic response to nerve stimulation might be interpreted as evidence for a role of endogenous adenosine as a feed back inhibitor of lipolysis. Further proof of this hypothesis is necessary however.

This study was supported by the Swedish Medical Research Council (proj. no 04X 2553) and by Karolinska Institutet.

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Antilipolytic Effect of Adenosine in Dog Adipose Tissue *in Situ*

By

BERTIL B FREDHOLM and ALF SOLLEVI

Adenosine and related compounds are released from isolated rat fat cells (see Schw *et al* 1975) and from canine subcutaneous adipose tissue (Fredholm 1976). The rate release from the latter preparation is enhanced by sympathetic nerve stimulation. Adeno inhibits cyclic AMP accumulation in isolated rat fat cells already in submicromolar c centrations (Fain *et al* 1972, Schwabe *et al* 1975) while lipolysis is inhibited to a consider lower degree in this preparation As discussed by Fain (1973) this apparent contradic might be explained if cyclic AMP levels are raised to supramaximal levels by the concen tion of the lipolytic drugs commonly used in studies of isolated fat cells. In a recent stud found that maximal rates of lipolysis in canine subcutaneous adipose tissue induced by n stimulation are associated with only small (50%) increases in tissue cyclic AMP co (Fredholm *et al* 1976)

In the present study we have therefore investigated the effect of adenosine on lipo induced by sympathetic nerve stimulation. A preliminary account of some of these has been given (Fredholm 1975)

21 fed, female mongrel dogs weighing 8–16 kg were used Anesthesia was induced ith 30 mg kg pentobarbital i v and supplemented as necessary during the expt. S bcutaneous adipose tissue as be (Rosell 1966) nd a toperfused. Responses were studied in 30 fat pads weighing 20–42 g The an were tracheotomized artificially ventilated and arterial pO_2, pCO_2 and pH controlled Heparin (1 kg) was given to prevent clotti g. Adipose tissue blood flow was measured using a drop counter nerve to the preparation was isolated and timulated at 4 H (2 ms, 12 V). Arterial and venous samples were draw and glycerol (Laurell and Tibbling 1966) was assayed on deproteinized plasma. A sine was administered by close i a. infusion. For futher experimental details see below Dipyrid (Persantin Boehringer Ingelheim) wa administered i

Adenosine infused i.a. to give concentrations in the arterial blood flow ranging bet 0 01 and 40 μM had no significant effect on the basal rate of glycerol release. In cont glycerol release evoked by electrical nerve stimulation was inhibited by adenosine in centrations above 1 μM (Table I)

In order to test the possible significance of endogenous adenosine the well-known ad sine uptake inhibitor dipyridamol (Schrader *et al* 1972) was administered to the dogs. results of these expts. are summarized in Table II While ineffective on basal glycerol re dipyridamol (0.5 mg/kg i.v) inhibited stimulated glycerol release.

The present results demonstrate that exogenous adenosine is antilipolytic in canine cutaneous adipose tissue *in situ* A 70% inhibition was found by administering adenos

TABLE I Inhibition of stimulated glycerol release by adenosine. $\bar{x} \pm S_{\bar{x}}$.

[illegible] concentration	Glycerol release (% of control)
3 (4)	100 ± 6
6-0 15 (6)	101 ± 6
3 (6)	117 ± 14
[illegible]	74 (61, 87)
40 (5)	27 ± 10 (p 0 001)

Stimulated glycerol release was calculated by summing the total amount of glycerol released during and following nerve stimulation and subtracting the basal release for the same overall time period.

TABLE II Effect of dipyridamol (Persantin®) on glycerol release from canine subcutaneous adipose tissue $\bar{x} \pm S_{\bar{x}}$

	Before dipyridamol	*After dipyridamol*[a]	Per cent of control
Basal release rate (µmol min⁻¹ 100 g⁻¹)	0 133 ± 0 026 (11)	0.156 ± 0 026 (7)	100 ± 16
Stimulated release (µmol 100 g⁻¹)	21 6 ± 3 4 (11)	13.3 ± 3 6 (7) p 0 01	57 4 ± 10 6 (7) p 0.01[c]

[a] Dipyridamol was administered in doses of 0 5–1 mg/kg before nerve stimulation.
[b] Students t-test for means of two samples
[c] Students t-test for one sample

rate calculated to give 10–40 µM concentration in the blood. Since adenosine is rapidly inactivated in blood and tissue (Schrader *et al* 1972) the concentration of adenosine achieved at the fat cells is probably considerably lower In isolated rat fat cells noradrenaline stimulated lipolysis was inhibited by less than 25 per cent at 10 µM adenosine (Hjemdahl and Fredholm 1976) Thus adenosine is a far better inhibitor of lipolysis *in vivo* than *in vitro* Since adenosine probably acts by inhibiting cyclic AMP formation (Fain 1973 Schwabe *et al.* 1975 Hjemdahl and Fredholm 1976) this may be related to the fact that cyclic AMP increases in vivo are far smaller than those commonly studied *in vitro* (Fredholm *et al.* 1977).

The recent finding that adenosine inhibits noradrenaline release from several tissues including canine subcutaneous adipose tissue (Hedqvist and Fredholm 1976) could also partially explain the potent antilipolytic effect of adenosine under the present experimental conditions

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This study was supported by the Swedish Medical Research Council (proj no 04X 2553) and by Karo-

References

FAIN, J. N. Biochemical aspects of drug and hormone action on adipose tissue. *Pharmacol. Rev.* 1973, 67, 118.

FAIN, J. N., R. H. POINTER and W. F. WARD, Effect of adenosine nucleotides on adenylate cyclase, phosphodiesterase, cyclic adenosine monophosphate accumulation, and lipolysis in fat cells. *J. biol. Ch.* 1972, *247*, 6866–6872.

FREDHOLM, B. B. Adenosine modulation of cyclic AMP and lipolysis in adipose tissue. Sixth Int. Pharm. Meeting. 1975. Abstract.

FREDHOLM, B. B. Release of adenosine-like material from isolated perfused dog adipose tissue following sympathetic nerve stimulation and its inhibition by adrenergic α-receptor blockade. *Acta physiol. scand.* 1976, *96*, 422–430.

FREDHOLM, B. B., E. BELFRAGE and E. BLASCHKE, Changes in ATP and cyclic nucleotide levels during sympathetic nerve stimulation in canine subcutaneous adipose tissue in situ. *Acta physiol. scand.* In press.

HEDQVIST, P. and B. B. FREDHOLM, Effects of adenosine on adrenergic neurotransmission, Prejunctional inhibition and postjunctional enhancement. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharm.* 1976, *293*, 17–223.

HJEMDAHL, P. and B. B. FREDHOLM, Cyclic AMP-dependent and independent inhibition of lipolysis by adenosine and decreased pH. *Acta physiol. scand.* 1976, *96*, 170–179.

LAURELL, S. and G. TIBBLING, An enzymatic fluorimetric micromethod for the determination of glycerol. *Clin. chim. Acta* 1966, *13*, 317–322.

ROSELL, S. Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic nerve stimulation. *Acta physiol. scand.* 1966, *67*, 343–351.

SCHRADER, J., R. M. BERNE and R. RUBIO, Uptake and metabolism of adenosine by human erythrocyte ghosts. *Amer. J. Physiol.* 1972, *223*, 159–166.

SCHWABE, U., R. EBERT and H. C. ERBLER, Adenosine release from fat cells. Effect of cyclic AMP levels and hormone actions. *Adv. Cyclic Nucleotide Res.* (G. L. Drummond, P. Greengard and G. A. Robison eds), 1975, vol. *5*, 569–584.

Acta physiol. scand. 1977 99 257–260

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The Effect of Oxygen on Peripheral Airways

By

TAGE MOLS NIELSEN and OLE FIND PEDERSEN

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Abstract

…ELSEN, T M, and O F PEDERSEN. *The effect of oxygen on peripheral airways.* Acta physiol. scand. 1977 99 257–260.

…n normal young subjects four alveolar O_2-concentration levels ranging from about 7 to 95 were …tained by breathing different concentrations of oxygen in nitrogen at constant alveolar CO_2-con…ntration of about 5%. Maximum expiratory flows at 60% TLC were measured at each level and …rected for the influence of the expired gas density on flow. The corrected maximum flows …ere not …nificantly different, …hich is taken as evidence of no effect of oxygen on tone of peripheral bronchi.

…ey words: Bronchomotor effect of O_2, O_2 tension, maximum expiratory flow-volume curves

…t has previously been shown that hypocapnia causes a progressive decrease of maximal …xpiratory flow at midlung volume, indicating constriction of peripheral bronchi (Nielsen …nd Pedersen 1976 b). This effect is of significance in maintaining a normal ventilation/ perfusion ratio, as it may help to decrease wasted ventilation of poorly perfused areas in the lungs. Similarly the pulmonary vasoconstriction caused by hypoxia helps to decrease wasted perfusion of poorly ventilated areas (*cf* review by Hughes 1975).

Whether oxygen also is of significance in maintaining normal ventilation in relation to perfusion is not known, even though its influence on bronchomotor tone has been investigated earlier. A review of the literature has been given by Barer *et al* (1972) and by Widdicombe (1963). In normal subjects there seems to be no influence of hyperoxia on airway resistance (Butler *et al* 1960, Astin and Penman 1967). Hypoxia, however seems to cause a fall in specific airway conductance both in normal subjects (Sterling 1968) and in patients …th chronic bronchitis (Astin and Penman 1967).

These measurements of resistance and conductance are mostly influenced by conditions in larger airways, however, and do not give special information about the influence of O on smaller airways, where the effector site must obviously be, if oxygen should be effective in changing local ventilation in relation to perfusion. The effect here of hyperoxia should then be bronchoconstriction and the effect of hypoxia bronchodilatation, which is apparently not the case for the total airway (*cf* above).

In the previous study we showed that hypocapnia caused a progressive decrease of t maximal expiratory flow at 60% total lung capacity (V_{max} (60% TLC)). The purpose of t present investigation has been to examine the effect on V_{max} of varying alveolar oxyg tensions (P_AO_2) under conditions with unchanged alveolar carbon dioxide tensions (P_ACO By using the same subjects as previously and, so far as possible, the same experime procedure, it should furthermore be possible to compare the effect, if any of changing P_A with the effect of changing P_ACO_2, both within physiological ranges.

Material and methods

Ten young healthy subjects (two females and eight males) were investigated. None of them had a hi of heart or lung disease. The subjects were the same as presented in an earlier paper (Nielsen and Pede 1976 b).

The experimental set-up and the procedure were with a few exceptions identical with those used in previous investigation and have previously been described in detail. Therefore only a brief account given here.

In a randomised way four series of maximum effort flow-volume (MEFV) curves were made for subject each series with different alveolar oxygen concentration (F_AO_2) produced by letting the sub respire gas mixtures with 12.51 20.95, 29.25 and 100% O_2 in N_2 in series I, II, III, and IV respectiv In each series five blows were made.

As in the previous study end-tidal CO_2 concentrations (F_ACO_2) were measured continuously they displayed for the subjects, and these were asked to ventilate to $F_ACO_2 = 5$ %. In the present study average of F_AO_2 of the last 10 expirations before the blow was furthermore measured by taking out 10 of gas, 10 ml at time, from the expiratory tube during the inspiration. This gas was passed throug oxygen analyzer (Servomex, OA 150), which was calibrated with known gases and show to gi response linearly related to the O_2 percentage.

On the MEFV curves, displayed on a storage oscilloscope and photographed with polaroid cam V_{max} (60% TLC) was measured, converted to BTPS, and corrected for the influence of density using formulas described previously (Nielsen and Pedersen 1976 a)

The corrected V_{max} (from hereon called V_{max} only) from the 4 series and the 10 subjects we compared by a two-way analysis of variance (anova) with replication (Sokal and Rohlf 1969), using same procedures nd significance levels as in the previous study

Results

In Table I are presented mean V_{max} and standard deviations for each series and subje It is seen that the individual differences between the series are small and that the differe between the grand mean for each series is indeed very small. This is confirmed by the anc which shows that there is no significant difference between the four series, the ratio of me squares (F_s) being 1.29 with 3 and 27 degrees of freedom (df). The difference between su jects is highly significant however F being 1169.39 with df = 9 and 160

It was possible to avoid an influence of CO on $\dot{V}_{max}$. The grand means of F_ACO for subjects in series I II III and IV respectively were 4 82%, 4 89%, 4 85%, and 4 81 These values were not significantly different from each other as F was 0.78 with df and 27

The mean values of F_AO in series I II III and IV respectively were 7.24 %, 15 17 23.87%, and 95 09%.

I. The mean of the corrected $\dot{V}_{max}$ (cf. text) for each subject in the 4 series. In brackets are shown the standard deviations. For comparison is shown the grand mean of uncorrected $\dot{V}_{max}$ in addition.

	Series I (F_IO_2 12.51 %) $\dot{V}_{max}$ ($l \cdot s^{-1}$)	Series II (F_IO_2 = 20.95) $\dot{V}_{max}$ ($l \cdot s^{-1}$)	Series III (F_IO_2 = 29.25 %) $\dot{V}_{max}$ ($l \cdot s^{-1}$)	Series IV (F_IO_2 = 100) $\dot{V}_{max}$ ($l \cdot s^{-1}$)
	3.13 (0.07)	3.36 (0.13)	3.25 (0.13)	3.06 (0.14)
	4.62 (0.30)	4.65 (0.07)	4.40 (0.13)	4.38 (0.13)
	3.32 (0.25)	3.15 (0.20)	3.16 (0.26)	3.29 (0.09)
	6.06 (0.22)	5.99 (0.21)	3.83 (0.34)	5.92 (0.30)
	7.26 (0.08)	7.47 (0.18)	7.39 (0.18)	7.49 (0.14)
	6.52 (0.18)	6.64 (0.26)	6.69 (0.29)	6.37 (0.13)
	4.58 (0.23)	4.76 (0.35)	4.61 (0.37)	4.73 (0.13)
	3.89 (0.30)	3.91 (0.24)	4.02 (0.21)	3.73 (0.03)
	2.85 (0.13)	2.84 (0.13)	2.79 (0.13)	2.70 (0.09)
	3.71 (0.25)	3.58 (0.17)	3.61 (0.13)	3.68 (0.07)
nd mean	4.59	4.63	4.58	4.54
nd mean of corrected $\dot{V}_{max}$	4.65	4.65	4.58	4.33

Discussion

e results thus indicate that changing F_IO_2 from about 7 to 95 % in normocapnic conditions does not have any influence on $\dot{V}_{max}$ (60% TLC), when correction is made for the fluence of different density of the exhaled gases.

In Table I the grand mean of uncorrected $\dot{V}_{max}$ for each series is shown. The difference tween the highest and lowest values is about 7 % compared to a difference of about 2 % f the grand means of corrected $\dot{V}_{max}$ (Table I). As it could be shown by anova that the fference between series of the uncorrected $\dot{V}_{max}$ was highly significant, it is of importance o use the correction when changing F_IO_2 to avoid a mistake that would have supported he hypothesis that F_IO_2 is of significance in maintaining ventilation in relation to perfusion cf. above).

Compared with the effect of hypocapnia (reducing normal P_ACO_2 by a factor 2 caused decrease of $\dot{V}_{max}$ by 14 %) the influence of O_2 on bronchial muscular tone can only be small. This does, however not completely exclude a small effect, which would have been evealed, had the method been more sensitive.

Our findings agree with those of Butler *et al.* (1960) who found no effect of hyperoxia on airway resistance in healthy subjects whether the effect was of short duration or of 5 to 6 h duration.

Astin and Penman (1967) as well found no effect on airway resistance, corrected for the influence of thoracic gas volume, of inhalation of 30 % O_2 in normal subjects, but they found 20 % decrease of resistance in patients with chronic bronchitis. This decrease was correlated to the hypoxemia of the patients before the administration of O_2. Astin (1970) confirms that the decrease in resistance after 30 % O_2 in such patients is related to the hypoxemia before the administration, and not to arterial carbon dioxide tension or arterial pH. Thus chronic hypoxemia in such patients causes bronchoconstriction.

In the previous study we showed that hypocapnia caused a progressive decrease of the maximal expiratory flow at 60% total lung capacity ($\dot{V}_{max}$ (60% TLC)). The purpose of the present investigation has been to examine the effect on $\dot{V}_{max}$ of varying alveolar oxygen tensions (P_AO_2) under conditions with unchanged alveolar carbon dioxide tensions (P_ACO_2). By using the same subjects as previously and, so far as possible, the same experimental procedure, it should furthermore be possible to compare the effect, if any, of changing P_AO_2 with the effect of changing P_ACO_2, both within physiological ranges.

Material and methods

Ten young healthy subjects (two females and eight males) were investigated. None of them had a history of heart or lung disease. The subjects were the same as presented in an earlier paper (Nielsen and Pedersen 1976 b).

The experimental set up and the procedure were with a few exceptions identical with those used in the previous investigation and have previously been described in detail. Therefore only a brief account is given here.

In a randomised way four series of maximum effort flow-volume (MEFV) curves were made for each subject, each series with different alveolar oxygen concentration (F_AO_2) produced by letting the subject respire gas mixtures with 12.51, 20.95, 29.25, and 100% O_2 in N_2 in series I, II, III and IV respectively. In each series five blows were made.

As in the previous study end-tidal CO_2 concentrations (F_ACO_2) were measured continuously, displayed for the subjects, and these were asked to ventilate to $F_ACO_2 = 5$ %. In the present study the average of F_AO_2 of the last 10 expirations before the blow was furthermore measured by taking out 100 ml of gas, 10 ml at a time, from the expiratory tube during the inspiration. This gas was passed through an oxygen analyzer (Servomex, OA 150), which was calibrated with known gases and shown to give a response linearly related to the O_2 percentage.

On the MEFV curves, displayed on a storage oscilloscope and photographed with a polaroid camera, $\dot{V}_{max}$ (60% TLC) was measured, converted to BTPS, and corrected for the influence of density using formulas described previously (Nielsen and Pedersen 1976 a).

The corrected $\dot{V}_{max}$ (from hereon called $\dot{V}_{max}$ only) from the 4 series and the 10 subjects were compared by a two-way analysis of variance (anova) with replication (Sokal and Rohlf 1969), using the same procedures and significance levels as in the previous study.

Results

In Table I are presented mean $\dot{V}_{max}$ and standard deviations for each series and subject. It is seen that the individual differences between the series are small and that the difference between the grand mean for each series is indeed very small. This is confirmed by the anova which shows that there is no significant difference between the four series, the ratio of mean squares (F_s) being 1.29 with 3 and 27 degrees of freedom (df). The difference between subjects is highly significant, however, F_s being 1169.39 with df = 9 and 160.

It was possible to avoid an influence of CO_2 on $\dot{V}_{max}$. The grand means of F_ACO_2 for the subjects in series I, II, III and IV respectively were 4.82%, 4.89%, 4.85%, and 4.8[illegible]%. These values were not significantly different from each other as F_s was 0.78 with df [illegible] and 27.

The mean values of F_AO_2 in series I, II, III and IV respectively were 7.24%, 15.1[illegible]%, 23.87%, and 95.09%.

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Renal Hemodynamics in the Perinatal Period

A Study in Lambs

By

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Abstract

peria, A., O. Broberger, P. Herin and I. Joelsson. *Renal hemodynamics in the perinatal period. A study in lambs.* Acta physiol. scand. 1977 99 261 269

e perinatal changes in renal hemodynamics have been studied in lambs. Eleven of the lambs were exterio-
ed, but maintained on placental circulation (fetal lambs). Eight of the lambs were delivered with cesarean
ction and studied immediately after clamping of the cord (newborn lambs). Nine lambs were delivered
ontaneously and studied during the first 9 days of life. Renal blood flow (RBF) was determined by the
icrosphere method using sample drawn from the iliac artery as the reference flow. The filtering capacity
f the nephrons was evaluated after ferrocyanide injection and dissection of the nephrons. Total RBF did
ot seem to change much at birth but increased in relation to kidney weight during the first postnatal week.
lamping of the cord did, however, result in change in intrarenal blood flow distribution, so that in
e newborn lambs relatively more of the blood flow was perfusing the outer cortical region. During the
rst postnatal week there was slight, but insignificant further relative increase in outer cortical blood flow
he relative increase in outer cortical blood flow at birth was accompanied by an increased frequency of
ltering superficial nephrons from 22 to 77 %. Practically all juxtamedullary nephrons were filtering before
irth. Three days postnatally 93 % of the superficial and 100 % of the juxtamedullary nephrons were filtering.

The fetal kidneys are known to produce urine already during the second third of gestation in those mammals where intrauterine recordings have been performed (Alexander and Nixon 1961). The degree of renal maturation reached at birth appears to be inversely related to the homeostatic efficiency of the placenta. In all species, however the placenta is the chief homeostatic organ during intrauterine life.

The aim of the present study has been to describe by which means the kidney will meet the requirements of extrauterine life. Special regard has been paid to renal hemodynamics since renal blood flow is of outmost importance for the postnatal renal functional development (Aperia *et al.* 1974). Total renal blood flow intrarenal blood flow distribution and the filtering capacity of the nephrons have been determined in lamb fetuses just before term, in newborn lambs immediately after the umbilical cord was clamped and in 1–9 days old lambs

Material and Methods

General preparation. The experiments were performed in twenty-eight lambs. Nineteen of those lambs were delivered with cesarean sections and studied as fetuses or newborns. In eight lambs the cord was

In normal subjects, Sterling (1968) found 22% fall in specific conductance of the
after inhalation of 10–12% O_2.

Compared to these results ours indicate that there is no influence of neither hypo
hyperoxia on tone in peripheral airways in normocapnic normal young subjects. This
necessarily in contrast to those of Sterling, but rather provides the new information th
change in conductance found by him is due to a change of tone in larger bronchi and tr
and not of tone in smaller bronchi

References

ASTIN, T W The relationships between arterial blood oxygen saturation, carbon dioxide tens
pH and airway resistance during 30 per cent oxygen breathing in patients with chronic bronchi
irway obstruction. *Amer Rev resp Dis* 1970. *102* 382 387

ASTIN T W and R W B. PENMAN Airway obstruction due to hypoxemia in patients with chro
disease. *Amer Rev resp Dis* 1967 *95* 567 575

BARER, G R., T W ASTIN, J W SHAW and P M WARREN, Bronchomotor effects of repeat
Bull Physio-path resp 1972. *8* 459–465.

BUTLER, J C. G CARO, R ALCALA and A. B. DU BOIS, Physiological factors affecting airway resi
normal subjects and in patients with obstructive respiratory disease. *J clin. Invest* 1960. 39. 3

HUGHES, J M B Cournand Lecture Efficiency of gas exchange in the lung. *Bull. Physio-path res*
11 921 935

NIELSEN T M and O F PEDERSEN A method to correct for the influence of gas density on
expiratory flow rate. *Acta physiol scand* 1976 a 98 123–130.

NIELSEN, T M and O F PEDERSEN, The effect of CO on peripheral airways. *Acta physiol scand*
98 192 199

SOKAL, R R. and F J ROHLF *Biometry The principles and practice of statistics in biological re*
W H Freeman and Company San Francisco. 1969 pp. 301 320, and pp. 369–375.

STERLING G M The mechanism of bronchoconstriction due to hypoxia in man. *Clin. Sci.* 19
105–114

WIDDICOMBE, J G Regulation of tracheobronchial smooth muscle. *Physiol. Rev* 1963. 43. 1 3

I General hemodynamic measurements. Oxygen tension (PO_2) and mean arterial blood pressure.

	Fetus	Newborn	1 3 days	6–9 days	31–48 days
mmHg	23.7 ± 3 50	42 0 ± 8 85	N.M.	N.M.	N.M
pressure, mmHg	64 1 ± 5 84	73 1 ± 5.94	86 3 ± 9.46	98.3 ± 10 8	107 1 ± 5 67

not measured.

...rtical and juxtamedullary nephrons were those defined by Boonvalst *et al.* (1972). The superficial ...uli would occupy the outer 10 and the juxtamedullary glomeruli the inner 15 % of cortical volume. ...four superficial and 29 juxtamedullary nephrons from ten fetal lambs, 35 superficial and 13 juxta-...llary nephrons from 6 newborn lambs and 16 superficial and 9 juxtamedullary nephrons from three ...ly old lambs were inspected Five of the 34 superficial nephrons in the fetal group were dissected ...into the distal tubule In most cases camera lucida drawing at the magnification 50– 80 ...each of the glomerulus and the entire proximal tubule, and the site of the ferrocyanide precipitate ...ined

...merular counting. Just before sacrificing the animals, 5–10 ml India-ink was given in the aorta just ... the renal arteries for color marking the glomeruli of the remaining right kidney The right kidney ...then removed and sectioned for determination of intrarenal blood flow distribution and number of ...uli. The kidney was divided transversely in an upper and lower half. One of the halves was ...only chosen for counting its total nuclide amount for the purpose of estimating total RBF The other ...was cut frontally in 2 3 about 1 mm thick sections. Each of these sections as sliced under dissection ...scope into an outer mid and an inner part, each occupying approximately 10–15 %, 60 %, and 25 ...rtical volume. Radioactivity was measured in gamma spectrometer (Packard autogamma spectro-...r) with lead shielded scintillation detector. In 5 fetal, 4 newborn and in 2 and 4 lambs of 1 3 and 6–9 ...of age, respectively slices from outer mid and inner cortex were also used for glomeruli counting ...ding to modified method by Damadian *et al.* (1965) The estimation of the density of perfused glome-...allowed calculation of the average blood flow to single glomeruli since it was assumed that all spheres ...trapped in the glomeruli. All renal tissue pieces were wet weighed before radionuclide and glomeruli ...ting.

The mean blood pressure was monitored by Statham transducer and recorded on a Grass model 7B ...graph The blood pressure did not decline during the experiment and was not significantly affected ...injection of microspheres and ferrocyanide. PO_2 was determined in arterial blood samples using Clark ...oxygen electrode. The values presented in Results were sampled immediately before injection of ...rospheres For statistical calculations, Student t-test has been used

Results

...he perinatal changes in arterial oxygen tension and mean arterial blood pressure are ...monstrated in Table I. Arterial oxygen tension (PO_2) was only recorded in the fetal and ...e newborn lambs. As expected, clamping of the cord resulted in a highly significant ...0.001) increase in arterial PO Clamping of the cord also resulted in an increase in ...rterial blood pressure that was significant ($p < 0.01$). The arterial blood pressure continued ...o increase almost linearly during the first 10 days of life. Thereafter the blood pressure ...ppears to have reached a steady level, since the mean pressure recorded in 31–48 days old ...mbs (Apula and Herla) is 107 1 ± 5 7 mmHg, which is not significantly different from ...he value recorded in 6–9 days old lambs ($p > 0.5$).

Fig. 1 shows the average changes in kidney weight and glomerular density during the perinatal period. The kidney weight was slightly lower in the fetal lambs than in the new-born lambs. During the first postnatal week there was approximately fifty per cent increase

clamped and the animals were allowed to breathe before the study (newborns). In addition, four 1-3 days old and five 6-9 days old lambs were studied. The body weights of the fetal and newborn lambs averaged 3 kg, of the 1-3 days old lambs 3.8 kg and of the 6-9 days old lambs 4.2 kg. The gestational age of the animals delivered with cesarian sections ranged between 135-145 days estimated from the date for artificial fertilization and maternal physical signs. Normal gestation time in sheep is 145-150 days. The pregnant ewes were allowed free access to food and water until 6 h before study. The ewes were given atropine (1 mg) and were anesthetized with a small dose of Pentothal sodium (6-7 mg/kg). Pancuronium bromide (Pavulon, 1.5 mg) was administered for muscle relaxation. The ewes were ventilated with known gas mixtures through an endotracheal tube connected to an Engström respirator. Arterial blood samples were taken repeatedly to control PO within adequate limits (80-120 mmHg) (Joelsson et al. ...). A 5 % glucose solution was continuously infused (150 ml/h) during the expt. Through a left paramedian incision the uterine wall was opened carefully to avoid placental bleeding. After delivery all lambs were placed on a thermoregulated table in order to maintain the initially recorded rectal temperature which ranged between 38.5-39.5°C. This is generally considered to be the optimal temperature of newborn lambs (Joelsson et al. 1970). After exteriorizing the fetus different procedures were used. In the fetal group the heads of the animals were immediately after exposure covered with a saline filled rubber glove to avoid air breathing. The umbilical cord was kept intact and prevented from stretching and cooling. In the newborn lambs spontaneous respiration was started immediately after the umbilical cord was clamped. In local anesthesia polypropylene (pp) catheters were inserted into the femoral arteries (pp 90). The tip of one femoral catheter was, during continuous pressure recordings, introduced into the left ventricle where it was permanently placed, or withdrawn to a place just above the aortic valves. The other femoral artery catheter was advanced only a few cm (iliac catheter). The preparation of the lamb described was completed within 8 min after delivery.

Lambs studied in the later postnatal period (four 1-3 days and five 6-9 days of age) were anesthetized and artificially ventilated as described above for the ewes. The lambs were catheterized as the lambs in the fetal and newborn groups.

Renal blood flow determinations with microsphere method. Renal hemodynamics were studied by the microsphere method. Spheres with a diameter of 15.1±3.1 μm and labeled with a radionuclide or Ce^{141}) were suspended in dextran (10 %) with Tween 80 added. A homogenous solution was obtained by ultrasonic treatment (Branson Sonic Power Company). Immediately thereafter 0.6-0.8 ml of the sphere solution or total number of around 700 000 microspheres was injected during about 15 s. The injection site in the left ventricle or just above the aortic valves should provide adequate mixing (... et al. 1973, Kleinman and Reuter 1973). Microspheres with a diameter of 15 μm are thought to be distributed within the kidney according to the renal blood flow and will almost exclusively be trapped in the glomerular capillaries during the first renal circulation (Buckberg et al. 1971, Katz et al. 1971). The number of spheres in renal tissue will therefore be proportional to blood flow in corresponding areas.

The absolute intrarenal blood flow was measured by means of reference blood sample (Archie ... 1973). This sample was withdrawn by a constant speed pump (3 ml/min) (Sage Instruments) from the iliac artery during 25 s from time of microsphere injection. As no blood is added to the aortic blood flow between the entrance of the renal arteries to the sampling site, there should be similar concentrations of spheres in blood perfusing the kidneys as in the reference sample. The ratio of nuclide amount in tissue and blood flow to corresponding tissue area would then be in proportion to the ratio of amount in the reference blood sample and the sampling rate (i.e. 3 ml/min).

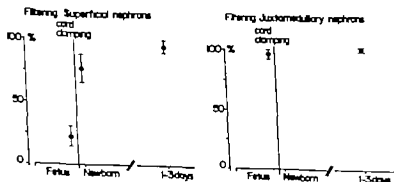
Determination of filtering capacity. The ability of the glomeruli to filter was determined with ferrocyanide as an indicator. The left kidney was exposed through a flank incision. 0.8 ml of a 10^{-} ferrocyanide solution was rapidly injected into the aorta just above the renal arteries. The transit time from the injection site to the glomerular capillaries had previously been determined (range 1-3 s) by inspecting the renal surface following the injection of 0.2 ml of 10 % solution of lissamine green. After 15 s plus the transit time following the ferrocyanide injection the renal pedicle was abruptly clamped. The kidney was then immediately excised and frozen in fluid nitrogen. The ferrocyanide remaining in the kidney was precipitated as Prussian blue in ferrichloride solution at −21°C during 18 h. This procedure was followed by maceration in 20 % HCl at 37°C for 4.75 h. The macerated kidney was kept in preservation solution (... in 95 ml ethanol and 5 ml conc. HCl) at +4°C until dissected. Complete superficial and juxtamedullary proximal tubules including pars recta were dissected under stereomicroscope, placed in a drop of water and inspected for the presence of Prussian blue particles, i.e. the ferrocyanide precipitate. ... of filtering glomeruli the ferrocyanide should be entirely recovered in the tubules. Only those ... were used where the ferrocyanide precipitate was intensely blue and could easily be observed. The

:II. Absolute values (mean ± S.D.) of renal hemodynamics.

	Renal blood flow ml/g renal tissue /min	Glomerular blood flow nl/min outer cortex	mid cortex	inner cortex
(n 5)				
m	1.55	23.2	21.8	31.6
)	±0.25	±6.46	±2.95	±6.43
orn (n=4)				
m	1.61	34.5	22.3	28.5
).	±0.48	±10.85	±5.80	±6.86
ys (n=4)				
m	1.75	78.8	63.8	39.8
)	_0.47	±22.4	±5.91	±8.66
ys (n 5)				
m	1.91	93.2	61.6	50.0
).	±0.43	±23.8	±18.4	±13.0

e newborn group. Postnatally however RBF/g will increase from birth to 6–9 days fe. This increase in RBF/g is quite remarkable, since at the same time there is also a increase in kidney weight.

he blood flow to the superficial glomeruli increased at birth both in absolute terms ble II) and in relation to the blood flow to the juxtamedullary glomeruli (Fig.). The between inner and outer cortical (IC/OC) tissue flow was obtained in all twenty-eight ts studied. The IC/OC ratios of GBF and tissue flow were significantly lower in the born than in the fetal lambs (p < 0.001 and p 0.01 respectively). During the first post al week there was slight further fall in the IC/OC ratio of GBF and tissue flow The es obtained in the newborn lambs were, however not significantly different from those rded in the 6–9 days old lambs (p > 0.5). Nor were they significantly (p > 0.5) different n those recorded in the 40 days old lambs (Aperia and Herin). In fact, the IC/OC ratio he 40 days old lambs was slightly higher than the ratio in the 6–9 days old lambs.



g. 3. The relationship between the number of filtering nephrons and the number of superficial nephrons ig. 3) and juxtamedullary nephrons (Fig. 3 b) studied. The dots represent the mean value in each age oup and the bars one standard deviation.

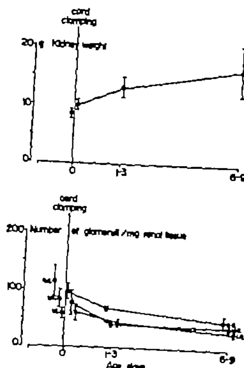


Fig. 1 Right kidney weight and mean number of gl
eruli counted per mg renal tissue (glomerular densi
outer (OC), mid (MC), and inner (IC) cortex in f
newborn, and in 1 3 and 6-9 days old lambs. The b
represent one standard deviation.

in kidney weight. The increase in kidney weight was accompanied by a fall in glomeru
density in all cortical layers. The glomerular density in the 6-9 days old lambs was s
nificantly lower than in the newborn lambs in outer cortex (OC) ($p < 0.001$), mid cor
(MC) ($p < 0.02$) as well as in inner cortex (IC) ($p < 0.01$)

The absolute values for total renal blood flow per gram kidney weight (RBF/g) and glom
ular blood flow (GBF) are given in Table II The results strongly suggest that total RB
will not change much at birth. The RBF/g values were practically identical in the fetal a

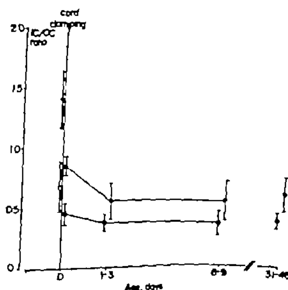


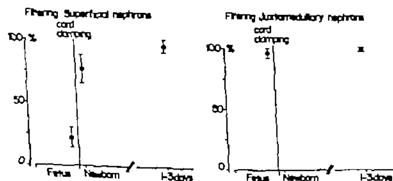
Fig. 2 The relationship between inner to
outer cortical (IC/OC) tissue blood flow ( )
and glomerula blood flow (GBF) (•) in
fetal, newborn and in 1 3 6-9 and 31-40
days old lambs. The dots represent the mean
value in each age group and the bars one
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	Renal blood flow, ml/g renal tissue /min	Glomerular blood flow nl/min outer cortex	mid cortex	inner cortex
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3. The relationship between the number of filtering nephrons and the number of superficial nephrons . 3 a) and juxtamedullary nephrons (Fig. 3 b) studied. The dots represent the mean value in each age p and the bars one standard deviation.

Fig. 3 a and b demonstrate the perinatal changes in the filtering capacity of the nephrons. The nephrons selected were localized with their glomeruli only in the superficial and juxtamedullary cortex. Practically all juxtamedullary nephrons were filtering before birth and remained filtering after birth (Fig. 3 b). The superficial nephrons, however displayed a large functional heterogeneity prenatally (Fig. 3 a) In each of the ten fetal lambs 3–12 superficial nephrons including complete proximal tubules with attached glomeruli were dissected free. In 4 of the fetal lambs none of the superficial nephrons inspected were filtering, in three of them less than 25% and in the remaining 3 fetuses, 33–70% of the superficial nephrons were filtering. Of the 39 nonfiltering nephrons, 5 were dissected and inspected to the distal tubules to assure that the bolus had not already passed the proximal tubule. In another inspected superficial nephrons (not included in the general calculations) Prussian blue was only located in the glomeruli In each of the 6 newborn lambs 3–10 superficial nephrons were inspected. In 3 of these lambs, all superficial nephrons were filtering. In the remaining three newborn lambs, 33 50 and 80% of the superficial nephrons were found to be filtering. In the three 1–3 days old lambs, 16 superficial and 9 juxtamedullary nephrons were dissected and of these, 94 and 100%, respectively were found to be filtering.

Since the time between the injection of the ferrocyanide and the arrival of the bolus in the glomerular capillaries was most likely not comparable in the different animals, no attempts have been made to make quantitative comparisons of the filtration in the different groups. It might be noted however that the mean distance between the ferrocyanide precipitate and the glomerulus was in each animal 10–15% larger in the filtering juxtamedullary than in the filtering superficial nephrons.

Discussion

Several methodological problems make interpretation of studies from the perinatal period difficult. The major problems include large individual variations, instability of preparations and large rapid changes in systemic circulation The large individual variations will in part depend on different conditions in fetal life. The fairly large scatter of the RBF values found both in this and in previous studies on fetal lambs (Beguin *et al* 1974 Rudolph and Heymann 1967) is therefore not surprising. The renal blood flow values recorded in the fetal lambs were, however in fairly good agreement with those observed previously (Beguin *et al* 1974 Rudolph and Heymann 1967)

The mean total RBF value in the fetal lambs did not differ significantly from the mean total RBF in the newborn lambs. This strongly suggests that total RBF will not change much at birth. During the first week, however there was a rise in the perfusion rate of renal tissue from 1 61 to 1 91 ml/g/min. In 31–48 days old lambs RBF/g averages 2.54 ml/g/min (Aperia and Herin).

Although cord clamping was not found to change total RBF it had a pronounced effect on intrarenal blood flow distribution The quotient between inner and outer cortical glomerular blood flow (IC/OC ratio) decreased significantly at the moment of birth. Such blood flow redistribution has previously been described in puppies but during a considerably longer period of time, *i.e* during the first 2 weeks of life (Kleinman and Reuter 1973).

the redistribution of intrarenal blood flow seems to be completed in the early postnatal In 2 79 days old lambs no significant change in inner to outer cortical tissue blood is been observed (Aperia *et al.* 1974). The fact that inner to outer cortical blood flow continues to fall during the first weeks of life in dogs (Jose *et al.* 1971 Kleinman and 1973, Olbing *et al.* 1973) is most likely due to different stages of renal maturation pies and lambs. The pronounced effect of birth on intrarenal blood flow distribution however well be a general process occurring in all species. The final effect will then d on the stage of renal maturation at birth. In lambs, the developmental changes of nal blood flow distribution will be almost completed at birth, while in dogs, birth only accelerate the centrifugal functional development.

rapid change in intrarenal blood flow distribution that occurs when clamping the might in part be due to the general circulatory changes that take place at birth (Assali 1962, Barcroft 1947 Dawes 1961). The arterial blood pressure increases significantly *et al.* 1970). In the adult animal it has been observed by several investigators (Källskog 1975, McNay and Abe 1970) that the outer cortical blood flow is more dependent on erfusion pressure than the inner cortical blood flow when the pressure is reduced below utoregulatory range, *i.e.* about 75 mmHg. In adult rats and dogs a decrease in arterial d pressure will thus result in higher inner to outer cortical blood flow ratio. A recent y from this laboratory (Aperia and Herin) in 2-9 days old lambs has shown that a ction in renal artery perfusion pressure to approximately 62 mmHg will cause a reduction e blood flow to the outer and mid cortex only The inner cortical blood flow will, ever, not be significantly changed when the pressure is reduced. During the first postnatal the arterial blood pressure will continue to increase but there will no longer be any ing effect on intrarenal blood flow distribution. It therefore seems likely that factors r than pressure are also responsible for the redistribution of intrarenal blood flow at h. The low arterial PO_2 before birth might contribute to the relatively low outer cortical usion in the fetal lambs since situations with hypoxia in the adult animals has been ribed to result in an increase of perfusion to the inner cortex (Barger 1966). The change intrarenal hemodynamics occurring at birth might of course also be due to vasoactive stances such as renin. At least the maternal concentration of this substance is high at the e of delivery and it is therefore believed to play an important role in the circulatory uption to extrauterine life (Mott 1975).

Clamping of the cord had dramatic effect on the filtering capacity of the superficial phrons. Glomerular filtration rate is determined by the product K_f P_{UF} K_f is the product the hydraulic conductivity and the surface area of the glomerular capillaries. P_{UF} is difference between the hydrostatic (P) and oncotic (π) pressure forces across the glomerular capillary. Since the effect of birth on the glomerular filtration in the superficial nephrons as immediate, it must be due to changes in P_{UF} rather than in K_f. The increase in P_{UF} n most likely be attributed to the combined effect of an increased hydrostatic pressure the glomerular capillaries (P_{GC}) and an increased glomerular plasma flow that will duce the mean oncotic pressure in the filtering glomerular capillaries (π_{GC}). Comparative udies in plasma loaded and hydropenic rats before and after aortic constriction have shown Robertson *et al.* 1972) that at the lowest recorded values for P_{GC} and glomerular plasma

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…llskog, M., Ö. Källskog and M. Wolgast. Effect of hemorrhage on single glomerular blood-flow as related to central hemodynamics and acid-base balance. *Acta anaesth. scand.* 1973, *17* 27–36.

…son, R. L., Micropuncture study of superficial and juxtamedullary nephrons in the rat. *Amer. J. Physiol.* 1970, *218*, 46–55.

…elson, J. M. D. Barton, S. Daniel, L. S. James and K. Adamsons, A method for prolonged monitoring of physiologic functions during fetal life. *Amer. J. Obstet. Gynec.* 1970, *107* 445–000.

…el, P. A., A. G. Logan, L. M. Slotkoff, L. S. Lilienfield, P. L. Calcagno and G. M. Eisner, Intrarenal blood flow distribution in canine puppies. *Pediat. Res.* 1971 *5*, 335–344.

…atz, M. A., R. C. Blantz, F. C. Rector, J. and D. W. Seldin, Measurement of intrarenal blood flow I. Analysis of microsphere method. *Amer. J. Physiol.* 1971 *220*, 1903–1913.

…inman, L. I. and J. H. Reuter, Maturation of glomerular blood flow distribution in the new born dog. *J. Physiol.* (Lond.) 1973, *228* 91–103.

…llskog, Ö., L. O. Lindbom, H. R. Ulfendahl and M. Wolgast. The pressure-flow relationship of different nephron populations in the rat. *Acta physiol. scand.* 1975, *94* 289–300.

…cNay, J. L. and Y. Abe, Pressure-dependent heterogeneity of renal cortical blood flow in dogs. *Circulat. Res.* 1970, *27* 571–587.

…ott, J. C., The place of the renin-angiotensin system before and after birth. *Brit. med. Bull.* 1975, *31* 44–50.

…ing, H. M. D. Blaufox, L. C. Aschinberg, G. L. Silkalns, J. Bernstein, A. Spitzer and C. M. Edelmann, Jr. Postnatal changes in renal glomerular blood flow distribution in puppies. *J. clin. Invest.* 1973 *52* 2385–2395.

…ertson, C. R., W. M. Deen, J. L. Troy and B. M. Brenner, Dynamics of glomerular ultrafiltration in the rat, III. Hemodynamics and autoregulation. *Amer. J. Physiol.* 1972, *223*, 1191–1200.

…udolph, A. M. and M. A. Heymann, The circulation of the fetus in utero. *Circulat. Res.* 1967 *21* 163–184.

…chmidt, U. and U. C. Dubach, Differential enzymatic behaviour of single proximal segments of the superficial and juxtamedullary nephron. *Z. ges. exp. Med.* 1971 *155* 345–353.

flow P_{oo} will be relatively important for the determination of P_{UF} When P_{oo} and glome plasma flow will increase the importance of the latter factor for the control of GFI increase. This is in accordance with the previous observation from this laboratory once the filtration is initiated its further development will closely follow that of the gl rular plasma flow (Aperia and Herin 1975)

The total RBF did most likely not change at birth. Yet, the intrarenal changes in b flow and function that occurred might have important consequences for total renal func The relative increase in the number of filtering superficial glomeruli strongly sugges increase of the total glomerular filtration from the moment of birth. The increased cont tion of the superficial nephrons to total renal function will also influence the work perfor by the kidney since there are functional differences between the superficial and the ju medullary nephrons both with regard to filtration (Baines 1973, Bonvalet et al 1972, Jam 1970) and tubular function (Bonvalet et al 1971 Schmidt and Dubach 1971).

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The authors are indebted to Mrs Ann-Christine Eklöf for expert technical assistance

References

ALEXANDER, D P and D A NIXON The foetal kidney Brit med. Bull. 1961 17 112 117

APERIA, A. and P HERIN, Development of glomerular perfusion rate and nephron filtration rate in c 17–60 days old Amer J Physiol 1975. 228 1319–1325.

APERIA, A. and P HERIN, Effect of arterial blood pressure reduction on renal hemodynamics in the develo ing lamb. A ta physiol. scand. 1976. 98 387 394.

APERIA, A., O BROBERGER and P HERIN, Maturational changes in glomerular perfusion rate and glomerul filtration rate in lambs. Pediatr Res. 1974. 8 758–765.

ARCHIE, Jr J P D. E. FIXLER, D. J ULLYOT J I. E. HOFFMAN, J R. UTLEY and E. L. CARLSON, Measure ment of cardiac output with and organ trapping of radioactive microspheres. Amer J Physiol 197 35 148–154

ASSALI, N S., N SEHGAL and S. MARABLE, Pulmonary and ductus rteriosus circulation in the fetal lamb before and after birth. Amer J Physiol. 1962. 202 536–540.

ASSALI, N. S., G H JOHNSON, C. R. BRINKMAN and T H KIRSCHBAUM, Control of pulmonary and systemic vasomotor tone in the fetus and neonate. Amer J Obstet Gynec 1970. 108. 761 772.

BAINES, A. D Redistribution of nephron function in response to chronic and acute solute loads. Amer J Physiol 1973 224 237 44.

BARCROFT J Researches on prenatal life. Springfield Thomas 1947

BARGER, A. C., Renal hemodynamic fact rs n congestive heart failure. Ann. N Y Acad Sci 1966 139 276–284.

BEGUIN, F D R. DUNNIHOO and E J QUILLIGAN Effect of carbon dioxide elevation on renal blood flow in the fetal lamb in utero. Amer J Obstet Gynec 1974 119 630–637

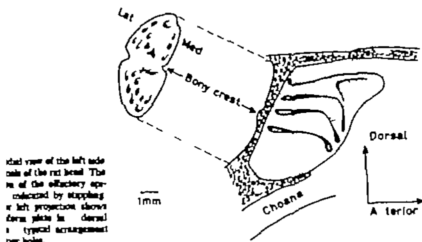
BONVALET J P P BENCSATH and C. DE ROUFFIGNAC, Glomerular filtration rate of superficial and deep nephrons during aortic constriction. Amer J Physiol 1972. ---- 599–606.

BONVALET J P C. DE ROUFFIGNAC and F MOREL, Relation débit/volume le long du tubule proximal du rein chez le rat. J. Physiol (Paris) 1971 63 117 A

BUCKBERG, G. D I C LUCK, D B. PAYNE, J I E. HOFFMAN, J P ARCHIE nd D E. FIXLER, Some sources of error in measuring regional blood flow with radioactive microspheres. J appl. Physiol. 1971 31 598–604

DAMADIAN, R V E. SHWAYRI and N S. BRICKER, On the existence of non-urine forming nephrons in the diseased kidney of the dog. J Lab clin. Med 1965. 65 26–38.

DAWES, G S., Changes in the circulation t birth. Brit med Bull. 1961 17 148–153



...dal view of the left side ...nals of the rat head. The ...n of the olfactory epi- ...indicated by stippling ...r left projection shows ...form plate in dorsal ... typical arrangement ...per holes.

...these observations and the observations on the spatial distribution of olfactory bulb ...es one can assume a non-homogeneous distribution of receptors within the epithelium. ...ultaneous recordings of the electro-olfactogram (EOG) at different sites of the sen- ...ithelium such a non-homogeneous distribution was demonstrated in frog (Musta- ...971 Daval, Leveteau and MacLeod 1969).

...wanted to compare the pattern of selective degeneration with a possible pattern of ...omogeneous distribution of receptor cells. In the present study we show spatial ...ution of the different types of receptor cells in the rat by analysis of the relative ampli- ...of the EOGs recorded from different areas of the cribriform plate.

Materials and methods

...tment of experimental animals was the same as used in the studies on the selective degeneration ...ing in the olfactory bulb of rat after prolonged odour exposure (Pinching and Doving 1974). White ...rats of the Wistar strain were used. They were kept in semi-germfree conditions in isolation ...ters to avoid rhinitis. The rats were between 180 and 230 g (10–23 weeks old) when used in the expts. ...experimental animal was anaesthetized with urethane given intraperitoneally (1.5 g per kg b wt). T... ...the sniffing action from the respiration tracheostomy was carried out and polyethylene ...ter was inserted through the tracheostoma into the choana. The animal was fastened in headholder ...skull was opened dorsally and the olfactory bulb and the frontal part of the cerebrum was removed ...pose the dorsal surface of the cribriform plate as indicated in Fig. 1 Lymph and cerebro-spinal ...was continuously removed by cellulose wicks. Deep body temperature of the preparation was main- ...d at 37.5°C. During unilateral recordings, the contralateral (right) nostril was plugged with vaseline.

...ding

...recording electrodes made contact with the basal side of the olfactory epithelium at the holes of the ...riform plate. Monopolar recordings were made against common indifferent electrode placed on the ...l. The electrodes were broken glass capillaries, tip diameter 25–100 μm, filled with agar Ringer and ...ried in electrode holders containing sintered Ag/AgCl (WPI, Hamden, Conn USA). The potentials ...e pre-amplified by DC-amplifier with input impedance of 10 MOhm, and recorded on pen recorder ...ckman R8 Dynograph 2 channel with DC-couplers, frequency response: DC to 20 Hz)

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From the Institute of Zoophysiology University of Oslo, Blindern, Norway

Spatial Distribution of the EOG in the Rat; a Variation with Odour Quality

By

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Received 1 July 1976

Abstract

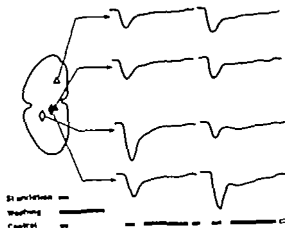
Thommesen G and K B. Døving *Spatial distribution of the EOG in the rat a va with odour quality* Acta physiol. scand. 1977 99 270-280.

The spatial distribution of olfactory receptors in the rat has been studied by simultaneous recording electroolfactograms (EOGs) from areas of the olfactory mucosa. The potentials were recorded f dorsal surface of the cribriform plate, leaving the mucosa and the nasal cavities i tact. The ratio betw peak amplitude of the EOG potentials served as a measure of the relative sensitivity of one area ag other The results obtained by stimulating with 31 substances at 38 positions demonstrate a non geneous distribution of different receptors. Each substance gives a spatial pattern of response eff The response distributions are mapped on the cribriform plate for the different odours.

The existence of different olfactory receptors in vertebrates has been demonstrated in (Gesteland *et al* 1963 Altner and Boeckh 1967 Duchamp *et al* 1974) and in the tor (Mathews 1972) A differential spatial distribution of receptor cells of different speci to olfactory stimuli is a possible basis for the functional organization of this sensory p way within the vertebrates. Adrian (1951) found that the different odours evoked maxi responses in different areas of the rabbit olfactory bulb. His findings were confirmed Mozell and Pfaffman (1954)

The spatial distribution of responses to odours within the olfactory system have also b demonstrated by other methods. Thus prolonged exposure to an odour causes a charac istic pattern of morphological changes named selective degeneration (Døving and Pinch 1973 Pinching and Døving 1974). Odour stimulation is associated with an increased me bolic activity which is unevenly spatially distributed (Sharp *et al* 1975).

Le Gros Clark (1951) made lesions in the glomerulus layer of the olfactory bulb of rabb On the basis of the retrograde degeneration patterns in the olfactory mucosa he conclud that the projection of the olfactory epithelium on the olfactory bulb was somatotopic. Th was confirmed by Land (1973) looking at the anterograde degeneration in the glomeru after sectioning of small bundles of the olfactory nerve. The projections showed howeve to be divergent and partially overlapping.



2. Differences in responses to [illegible] and methylbenzoate in positions of the mobile elec[trode] [illegible] the stationary electrode. [Elec]trode positions are shown to left. Stimulation time is 1 s. [Ve]rtical bar 0.5 mV

[illegible]

[Vari]ous forms of control expts. were performed. The stability of the preparation and the experimental [con]ditions were controlled by repeating the first stimulus at the end of each stimulation series. Expts. [we]re made to control the ratio N at various odour intensities. In 17 series several stimulations were made [wi]th the same odour at different intensities. These expts. would indicate possible systematic variations [of] the ratio N between the amplitudes at the stationary and mobile electrode positions with the concentra[tio]n of the odour. To control the variability from animal to animal eight pairs of presumed equivalent [re]cording positions were studied.

Results

The extension of the olfactory mucosa on the endoturbinal surfaces was studied in several rats and an average distribution is shown in Fig. 1. The difference between the sensory and the respiratory surfaces is clearly indicated by the ciliary movements in the latter epithelium in a freshly killed animal.

By recording from the dorsal surface of the cribriform plate as described by Ottoson (1954, 1959) the EOGs are recorded without interfering with the air flow in the nasal cavities. EOGs were positive when recorded from the basal side of the olfactory epithelium (Ottoson 1954, 1959) and behaved like a summated generator potential. If the electrode penetrated the mucosa, the EOG became negative.

The time course of the EOG agrees with that described by Ottoson (1954, 1956, 1959). After a latency of approximately 0.2 s the EOG rose to full size in 0.5 to 0.7 s. By prolonged stimulations an adaptation was seen whereby the EOG reduced to about 2/3 of its peak amplitude. Only the peak amplitude has been subject to analysis in the present article. EOG amplitudes to odorous stimulation varied from 0.2 to 2.5 mV. To standardize recording conditions, stimulation procedures were modified so that responses usually did not exceed 1 mV, thereby minimizing stimulation of receptor types with different specificity. Examples of the recordings are shown in Fig. 2.

TABLE I List of stimuli.

Order of stimulation	Symbols	Name	Order of stimulation	Symbols	Name
4	C5	Cyclopentanone	17	HOL	1 Heptanol
5	C6	Cyclohexanone	16	ION	β-Ionone
24	C7	Cycloheptanone	11	IBA	Isobornylacetate
6	C8	Cyclooctanone	27 31	IPA	Isopentylacetate
25	C9	Cyclononanone	8	LIM	(+)-Limonene
1	C10	Cyclodecanone	21	MEN	L Menthol
	C11	Cycloundecanone	13	MBZ	Methylbenzoate
26	C12	Cyclododecanone	22	MBL	2 Methyl-3-butyn-2-[illegible]
3	C13	Cyclotridecanone	27 31	MHN	6-Methyl-5-hepten-2-[illegible]
15	ACE	Acetophenone	23	MUL	2 Methylundecanal
27–31	BEN	Benzylthiol	10	MVA	4-Methyl valeric acid
20	CAM	DL-Camphor	9	NAF	Naphthalene
7	CHL	Cyclohexanol	19	NBZ	Nitrobenzene
18	DEC	1 Decanol	27 31	OCT	Octanal
12	EAA	Ethyl acetoacetate	14	MCC	1 1,2,2,-tetramethyl-3-dichloro-cycloprop[illegible]
27 31	HAL	Heptanal			

Stimulation

The catheter i the choana was connected to a suction pump via a flowmeter and a magnetic valve valve was controlled by a programming device giving 1 s suction, 1 pause, 5 s suction, ½ s pause suction Suction air flow was held t 7 14 ml/s. The preparation was continuously supplied with pu and humidified air through a glass funnel at a rate of 100 ml/s, producing a clean air-mask aroun snout.

I the studies of selective degeneration (Pinching and Døving 1974) were used 44 different odours the present investigation 31 of them were found to be potent enough to give basis for discussion odours and their order of presentation are listed in Table I. The stimuli were introduced on polyeth spatulae between the funnel and the snout just before the first (1 s) suction and withdrawn durin first pause. The second (5) suction thus served as washing and the last (½ s) suction as a contr every position the total series of stimuli were used Grading of stimuli was performed by stylin position of the spatula i the clean air flow

Experimental procedure

For positioning the recording electrodes on the preparation the horizontal bony crest dividin cribriform plate in two approximately equal parts, served as landmark. One electrode was placed c left cribriform plate in position approximately 0.5 mm ventral to this crest and 1 mm from the extension as seen in Fig. 2 and 3 This electrode will later be referred to as the "stationary electrode other recording electrode was successively placed at 38 different positions uniformly spread over th cribriform plate, and will be referred to as the mobile electrode Every preparation was photogr nd the electrode positions were plotted on the photograph. The different electrode positions are sho Fig 6 a.

Dat treatment

The treatment of the results is based on the method used by Mustaparta (1971). The ratio, *N* betwe the EOG amplitude of the mobile electrode nd that of the stationary one served as a measure fo stimulating efficiency of each substance t the position of the mobile electrode. This procedure was to minimize the effects of variable concentrations.

The results were transferred to non-parametric scale by ranking the ratios according to decreasing at every recording position. The response t the position of the stationary electrode was quantified mean rank value for each stimulant. This value is equal t 32 minus the mean of all the rank nu obtained for that stimulant and regarded as equivalent to the proper rank numbers. For each od substance the ranks were noted on position maps similar to those shown in Fig. 5 and 6.

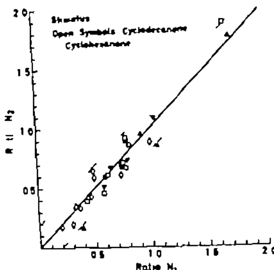


Fig. 4. The ratios N obtained by stimulation with the same substance at the beginning (N_1) and at the end (N_2) of each stimulation series. Graph symbols refer to the different preparations as indicated, Fig. 6 a. Broken lines show the 95 % confidence interval.

N varies from one electrode position to the other. The diagram demonstrates the general tendency of the ratio to be constant over a large range of concentrations for the same stimulus and electrode position.

In 32 runs covering several recording positions, 3–8 consecutive stimulations were made with the same substance. The standard deviation of the ratio N of the EOG amplitude at the mobile electrode to that at the stationary one, with the same substance and position, showed a mean value of 0.07. This standard deviation is less than that obtained for the total series of stimuli in any recording position, which ranged from 0.08 to 0.42, the lowest values in medial positions.

The stability of the preparation and the reproducibility of the results were also controlled by repeating the first stimulation at the end of each series. This was done in all cases except for the 4 ventral positions mentioned above. Fig. 4 shows the ratio N for the first (N_1) and the last (N_2) stimulation with the same substance in the 34 stimulation series. In all but one case the control stimulus was cyclodecanone. At three positions only the difference between the ratios fall outside a 95 % confidence interval found by using the above value of SD(N). At none of the positions included in this presentation the differences fall outside the 98 % confidence interval centered at zero. The test confirms that the preparations did not change significantly in sensitivity throughout the expts.

The results consist of measurements made on five animals, 4 to 12 recording positions in each (Fig. 6 a). The positions cover overlapping areas of the cribriform plate. Due to individual variations none of the positions could be regarded as completely congruent in different animals. Some recording positions were however assumed to be equivalent from one preparation to another, indicated by overlapping symbols in Fig. 6 a.

We have made eight comparisons, calculating the Spearman rank correlation coefficients. In seven of these comparisons the ranking of odours were positively correlated with signif

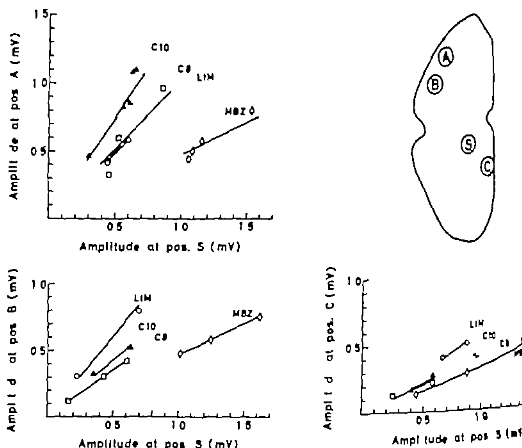


Fig. 3 The peak amplitudes of the electro-olfactogram recorded with different stimulus intensities. The recordings at the different positions A, B and C are simultaneous with those at the stationary electrode position, S. Pos. A and pos. B are from the same animal.

The abbreviations in the diagrams refer to the different chemicals listed in Table 1 The straight lines are drawn through the origin Note the difference in stimulation efficiency of limonene between pos. and pos. B compared with that of the cycloketones.

At four positions along the ventral margin of the cribriform plate, hatched on the position map Fig. 6 a, most responses were too faint to give substantial bases for data treatment.

Purified air elicited responses of 0 1–0 2 mV depending upon the flow of air suction presumably due to imperfect purification. This response disappeared only when the suction air flow was insufficient for odour stimulation.

Controls of the reliability of the data

Various expts. were performed to study the reliability of the results. As mentioned in the discussion the ratio *N* between two recording positions should be independent of stimulus intensity To test this hypothesis repetitive stimulations were made with varying stimulus concentrations. Fig. 3 shows the amplitudes of the EOG at the stationary electrode position versus those from 3 other areas. Plots have been made for four odours, *viz* two cycloketones, limonene and methyl benzoate. The ratio *N* is shown as the slope of the line through the origin. The variation is small with different concentrations but the slopes or the ra

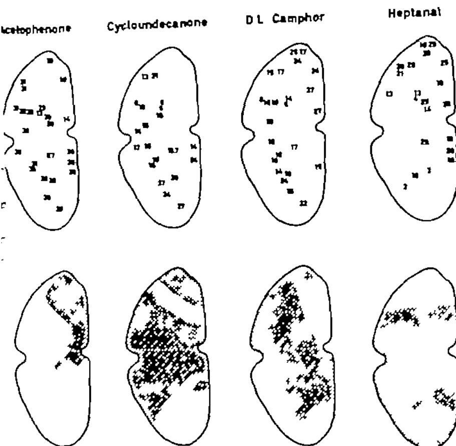


Fig. 5 Examples of response distributions to four different odours. Upper row: the rank numbers obtained at every position. Low rank numbers show high response ratio. Lower row: Simplified response distribution maps. Areas with rank numbers lower than 20 are hatched, areas with rank numbers lower than 10 are crosshatched.

response distribution of methylheptenone shows any similarity to that of camphor. The maps obtained for benzylthiole, cyclononanone, ethyl acetoacetate, isobornylacetate, isopentylacetate, limonene, methylundecanal, octanal and tetra-methyl-dichloro-cyclo-propane bear the closest resemblance to the heptanal map. The responses to β-ionone, methylbutynol and heptanol were so uniformly distributed that no pattern will be proposed.

The majority of 26 substances can also be seen to divide in two groups with response distribution maps approximately complementary to each other. One group of 16 odours represented by acetophenone and cycloundecanone, gives the strongest response dorsally or dorsomedially. The group of 10 here represented by heptanal give the strongest responses ventrally or laterally.

Another way of representing the results is shown in Fig. 6. In map of the cribriform plate the recording positions are marked with different symbols for each preparation (Fig.

icance levels less than 0.5 per cent. In only one comparison concerning positions centrally in the dorsal half of the cribriform plate there was no significant correlation. These correl- indicate that the results included in this presentation were reproducible from one prepara- tion to another.

Stimulating with different substances

At all the different positions studied the ratio of the EOG amplitude at the mobile rt the stationary electrode position varied with odour quality. Recordings of the EOG from differ- ent positions on the cribriform plate are shown in Fig. 2. Recording pairs with positions marked with the same symbols were recorded simultaneously. As seen limonene gave the smallest amplitude at the stationary electrode position in both cases. Methyl-benzoate gave the highest response at the "mobile" position in the upper pair but for the EOGs at the lower pair of positions, the highest response was at the stationary electrode. Relative shifts in stimulation efficiency are also demonstrated in Fig. 4 for limonene and two of the cyclo- ketones.

Comparisons of EOGs from left and right sides

3 series of simultaneous recordings have been made with the 2 recording electrodes sym- metrically placed on the left and the right side cribriform plate. In this case, the ratio *V* was found to be similar for all odours. The variations were not greater than expected from random variations in stimulus concentrations on the two sides, inaccuracy in response measurements or the uncertainty in finding exactly equivalent positions on nearly symmetri- cal structures. There is therefore no basis for assuming qualitative differences in sensitivity distribution between the olfactory epithelium of the right and the left side.

Mapping of response distribution

The *N* values of each position of the mobile electrode were ranked, giving the number 1 to the highest ratio and rank number 31 to the lowest. The rank numbers were plotted on the map of the cribriform plate, one map for each stimulus used. 4 examples of such maps are given in the upper row of diagrams in Fig. 5. In an attempt to visualize the sensitivity distri- bution in the olfactory epithelium as it appears by recording from the cribriform plate, the maps were simplified by hatching. Areas with rank numbers 1–9 have been crosshatched as a sign of high stimulus efficiency. Areas with rank numbers 10–19 have been covered with simple hatching and areas with rank numbers 20–31 are unhatched as signs of medium and low response respectively. In Fig. 5 this arbitrary and simplified visualization of the data is shown for acetophenone, cycloundecanone, camphor and heptanal.

The four maps represent 4 different types of distribution pattern which are fairly typical. Of the remaining 27 substances, 24 gave distribution maps which had visual similarity to one of the four maps shown. Similarity to the acetonphenone map was found for the lower cycloketones of 5–8 carbon atoms, cyclohexanol, menthol, methylbenzoate, 4-methyl valeric acid, naphthalene and nitrobenzene. Responses to decanol, cyclodecanone, cyclododecanone and cyclotridecanone were distributed similarly to the map for cycloundecanone. Only the

n variation in the electrical noise of the recording devices and the uncertainty in ng of the potentials.

OG may be recorded anywhere in the proximity of the olfactory epithelium. The nvenient recording site in the mammal is the nearly two-dimensional cribriform the described preparation procedure the EOG potentials are recorded without inter ith the nasal flow pattern since the nasal cavities are intact. The recordings are also lectrode potential artefacts as the electrode tips are not exposed to the stimulating nt.

corded potentials are electrotonic projections of potentials from a highly folded ol-epithelium serving as a volume conductor The potentials are not generated by the y axons projecting through the holes of the cribriform plate, and the exact location acted receptor cells within the epithelium can not be deduced from the distribution onses.

ne of these reservations it is reasonable to presume that the different parts of the ol-epithelium project mainly to nearby parts of the cribriform plate. For instance, a response in the dorsal area of the cribriform plate is most likely caused by activation tors mainly located in the roof of the nasal cavity or on the upper turbinates. Adrian suggested that the spatial differences in the bulbar responses to different odours were by non-homogeneous distribution of olfactory receptors of different specificity The etation assumed that the neuronal projection from the olfactory mucosa to the ol- bulb was more or less somatotopic as in fact was demonstrated the same year by s Clark (1951). Spatial differences in the distribution of different olfactory receptors onfirmed by Mustaparta in 1971 as evident in the frog.

tability of the distribution maps

present study several types of control experiments were performed, the variation of tio N at the same position for different odour intensities, the stability of one prepara-hroughout the expt. and the reproducibility from one animal to another The results se control experiments demonstrate the consistency of the reported data. They also te further repetitions of the experiments at homologous positions in different animals. ntamination of the odorous substances is another possible cause of error Aldehydes veral are susceptible to oxidation producing the corresponding acids, the effect of which have distorted the response distribution maps of the three aldehydes. The cyclononanone le is known to contain impurities, presumably di-ketones and aldehydes. It is therefore esting to note that cyclononanone, as the only one of the cycloketones, places itself e group of substances which also includes the three aldehydes.

Conclusions

present results show that receptor neurones of different spectra of sensitivity are differ ially distributed in the mammalian olfactory mucosa as well. In spite of the complex ding of this epithelium, the spatial distribution of receptor responses is clear None of

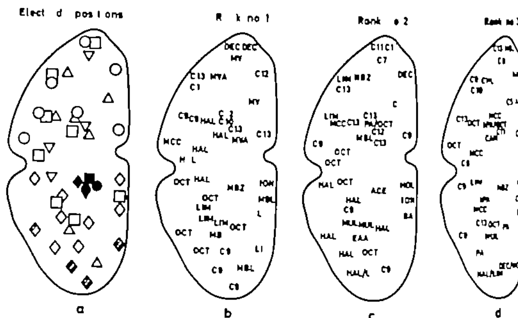


Fig. 6. Schematic diagrams of the left cribriform plate (see Fig. 1). *a* shows the different electrode positi
The different symbols refer to different preparations. Black symbols show the positions of the stand
electrode. Hatched symbols mark positions where only a few substances elicited a measurable respo
b, *c* and *d* show what substances obtained rank number 1, 2 and 3 respectively (see text)

6 a). In the following maps the odours are plotted which had the first, second and thi rank in each position. The maps show the same grouping of substances as mentioned abo and shown in Fig. 5.

Discussion

The general properties of the electro-olfactogram (EOG) in frog have been analysed i detail by Ottoson (1956). The objections raised by various authors concerning the features o the EOG and its relation to the receptor mechanisms have been discussed by Ottoson (1971 In the present study we have considered the EOG to represent the electrotonic projectio of the sum of generator potentials produced by those receptor cells which respond to th stimulus.

The laws for the spread of electrical potentials from a dipole layer in a volume conducto imply that the ratio between the potentials recorded from two different points is a functio only of the shape and location of the dipole layer but not of the voltage. In the present wor it is assumed that the recording of a sum of generator potentials from the olfactory mucos follows the same rule, as long as only one homogeneous population of olfactory receptors ar activated. From this assumption follows that during stimulation with different odours, th ratio between the EOG potentials recorded from 2 different points in the olfactory epitheliu will vary if and only if the epithelium contains receptor populations with different spectra of sensitivity and different spatial distribution. The ratio will be independent of the con centration of the stimulant within certain limits, *i.e.*, as long as receptor types with different thresholds are not activated, so that only one receptor population is involved. Even under ideal circumstances the ratio between the two recorded potentials will vary, however, due

random variation in the electrical noise of the recording devices and the uncertainty in reading of the potentials.

The EOG may be recorded anywhere in the proximity of the olfactory epithelium. The most convenient recording site in the mammal is the nearly two-dimensional cribriform plate. By the described preparation procedure the EOG potentials are recorded without interference with the nasal flow pattern since the nasal cavities are intact. The recordings are also free of electrode potential artefacts as the electrode tips are not exposed to the stimulating substances.

The recorded potentials are electrotonic projections of potentials from a highly folded olfactory epithelium serving as a volume conductor. The potentials are not generated by the olfactory axons projecting through the holes of the cribriform plate, and the exact location of the excited receptor cells within the epithelium can not be deduced from the distribution of responses.

In spite of these reservations it is reasonable to presume that the different parts of the olfactory epithelium project mainly to nearby parts of the cribriform plate. For instance, a strong response in the dorsal area of the cribriform plate is most likely caused by activation of receptors mainly located in the roof of the nasal cavity or on the upper turbinates. Adrian (1951) suggested that the spatial differences in the bulbar responses to different odours were · y non-homogeneous distribution of olfactory receptors of different specificity. The interpretation assumed that the neuronal projection from the olfactory mucosa to the olfactory bulb was more or less somatotopic as in fact was demonstrated the same year by Le Gros Clark (1951). Spatial differences in the distribution of different olfactory receptors were confirmed by Mustaparta in 1971 as evident in the frog.

The reliability of the distribution maps

In the present study several types of control experiments were performed: the variation of the ratio N at the same position for different odour intensities, the stability of one preparation throughout the expt. and the reproducibility from one animal to another. The results of these control experiments demonstrate the consistency of the reported data. They also obviate further repetitions of the experiments at homologous positions in different animals.

Contamination of the odorous substances is another possible cause of error. Aldehydes in general are susceptible to oxidation producing the corresponding acids, the effect of which may have distorted the response distribution maps of the three aldehydes. The cyclononanone sample is known to contain impurities, presumably di-ketones and aldehydes. It is therefore interesting to note that cyclononanone, as the only one of the cycloketones, places itself in the group of substances which also includes the three aldehydes.

Conclusions

The present results show that receptor neurones of different spectra of sensitivity are differentially distributed in the mammalian olfactory mucosa as well. In spite of the complex folding of this epithelium, the spatial distribution of receptor responses is clear. None of

the response distribution maps were identical. On the basis of their shape, the maps may [be] grouped, as belonging to a small number of patterns, some of which make up pairs of [...] complementary response distributions. A further analysis of the data on the similarity [of] response distributions will appear in a subsequent article. Difference in the distribution [of] the EOG does not indicate that there are discrete areas of uniform specificity. If a fi[xed] number of receptor types exists, the receptors most likely cover overlapping fields in t[he] olfactory mucosa.

The non-homogeneous distribution of olfactory receptors of different specificity impl[ies] that if two substances show different response distributions, they most likely are perce[ived] by the animal as being different. The reverse statement is not necessarily true. Recept[ors] sensitive to different odours may be intermingled so as to produce the same EOG distri[bu]tion.

This study has been supported by the Norwegian Research Council for Science and the Humanities.

References

ADRIAN, E. D. Olfactory discrimination. *Ann. psychol.* 1951, *50*, 107-113.

ALTNER, H. and J. BOECKH. Über das Reaktionsspektrum von Rezeptoren aus der Riechschleimhaut [von] Wasserfröschen (Rana esculenta). *Z. vergl. Physiol.* 1967, *55*, 299-306.

DAVAL, G., J. LEVETEAU and P. MACLEOD. Electroolfactogramme local et discrimination olfactive [chez] la Grenouille. *J. Physiol.* (Paris) 1969, *61*, Suppl. 2.

DØVING, K. B. and A. J. PINCHING. Selective degeneration of neurones in the olfactory bulb follow[ing] prolonged odour exposure. *Brain Res.* 1973, *52*, 115-129.

DUCHAMP, A., M. F. REVIAL, A. HOLLEY and P. MACLEOD. Odour discrimination by frog olfac[tory] receptors. *Chem. Senses and Flavor* 1974, *1*, 213-233.

GESTELAND, R. C., T. Y. LETTVIN, W. H. PITTS and H. ROJAS. Odour specificities of the frog's olfa[ctory] receptors. *Olfaction and Taste.* Pergamon Press, Oxford, 1963, *1*, 19-34.

LAND, L. J. Localized projection of olfactory nerves to rabbit olfactory bulb. *Brain Res.* 1973, [...] 153-166.

LE GROS CLARK, W. E. The projection of the olfactory epithelium on the olfactory bulb in the ra[bbit]. *J. Neurol. Neurosurg. Psychiat.* 1951, *14*, 1-10.

MATHEWS, D. F. Response Patterns of Single Neurons in the Tortoise Olfactory Epithelium [and] Olfactory Bulb. *J. gen. Physiol.* 1972, *60*, 166-180.

MOZELL, M. M. and PFAFFMANN, C. The afferent neural processes in odor perception. *Ann. N.Y. A[cad.] Science* 1954, *58*, 96-108.

MUSTAPARTA, H. Spatial Distribution of Receptor Responses to Stimulation with Different Od[ours]. *Acta physiol. scand.* 1971, *82*, 154-166.

OTTOSON, D. Sustained potentials evoked by olfactory stimulation. *Acta physiol. scand.* 1954, *32*, 384[...]

OTTOSON, D. Analysis of the electrical activity of the olfactory epithelium. *Acta physiol. scand.* 1956 [...] Suppl. 122, 1-83.

OTTOSON, D. Studies on slow potentials in the rabbit's olfactory bulb and nasal mucosa. *Acta ph[ysiol.] scand.* 1959, *47*, 136-148.

OTTOSON, D. The electro-olfactogram. A review of studies on the receptor potential of the olfa[ctory] organ. In *Handbook of Sensory Physiology.* Springer Verlag. 1971, *4*, 95-131.

PINCHING, A. J. and K. B. DØVING. Selective degeneration in the rat olfactory bulb following expos[ure to] different odours. *Brain Res.* 1974, *82*, 195-204.

SHARP, F. R., J. S. KAUER and G. SHEPHERD. Local sites of activity-related glucose metabolism i[n rat] olfactory bulb during olfactory stimulation. *Brain Res.* 1975, *98*, 596-600.

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Effects of Acetylsalicylate on Alkalinization, Acid Secretion and Electrogenic Properties in the Isolated Gastric Mucosa

By

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Abstract

...NER, A. *Effects of acetylsalicylate on alkalinization, acid secretion and electrogenic properties in the isolated gastric mucosa*, Acta physiol. scand. 1977 99 281 291

...effects of acetylsalicylate (ASA) on the *in vitro* secretory and electrical properties of *Necturus* and *Rana* ...pens gastric mucosa have been studied. The gastric antrum alkalinized the luminal surface, while ...he fundus it is likely that acidification and alkalinization occur simultaneously and that net secretion is ...to the dominance of one or other of these processes. The histamine H_2 receptor antagonist Metiamide ...used to inhibit acid secretion for studies on fundic alkalinization in *Rana temporaria*. Submucosal ...ication of 3 mM ASA for 30 min markedly reduced alkalinization in the antrum and the frog fundus. ...lowing removal of ASA there was only partial recovery of this secretion. The drug caused slight inhibi...n of spontaneous acid secretion in *Necturus* fundus but not of histamine-stimulated acid secretion in the ...g fundus. Following salicylate removal, the rate of acid secretion increased to higher level than before ...mulation in both tissues. There was small flux of ASA across the mucosa which was greatest in the ...d secreting frog fundus (4.30 μmol cm^{-2} min^{-1}) and least in the antrum (2.19 μmol cm^{-2} min^{-1}). Exposure ...the gastric mucosa to ASA was generally associated with fall in transmucosal electric potential differ...ce and short-circuit current together with an increase in electrical resistance. It is proposed that the greater ...sitivity of alkalinization to ASA is responsible for the apparent increase in the rate of acid secretion ...hich occurred.

Key words: Gastric mucosa, acetylsalicylate, alkalinization, acid secretion, electrogenic properties

Numerous clinical and laboratory studies have demonstrated that aspirin damages the gastric mucosa. A single oral dose can cause visible erosion of the epithelial lining of the stomach (Anderson 1964, Thorsen *et al.* 1968), an increase in the rate of exfoliation of gastric mucosal cells (Croft 1966) and gastric bleeding (Grossman, Matsumoto and Lichter 1961 Davenport 1969). Exposure of the mucosa to aspirin is also associated with changes in electrophysiological properties and permeability characteristics, both *in vivo* (Davenport 1964 Fromm 1971 Smith *et al.* 1971) and *in vitro* (Sachs, Hirschowitz and Shoemaker 1972, Flemström and Marsden 1973).

Assessment of the effects of aspirin on gastric acid secretion *in vivo* is complicated by the fact that inhibition of acid output *per se* is accompanied by an increase in mucosal perme-

the response distribution maps were identical On the basis of their shape, the maps can be grouped, as belonging to a small number of patterns, some of which make up parts of other complementary response distributions. A further analysis of the data on the similarity in response distributions will appear in a subsequent article. Difference in the distribution of the EOG does not indicate that there are discrete areas of uniform specificity If a limited number of receptor types exists, the receptors most likely cover overlapping fields in the olfactory mucosa.

The non-homogeneous distribution of olfactory receptors of different specificity implies that if two substances show different response distributions, they most likely are perceived by the animal as being different. The reverse statement is not necessarily true. Receptors sensitive to different odours may be intermingled so as to produce the same EOG distribution

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References

ADRIAN, E. D Olfactory discrimination. *Ann. psychol.* 1951 *50* 107 113.

ALTNER, H and J BOECKH, Über das Reaktionsspektrum von Rezeptoren aus der Riechschleimhaut von Wasserfröschen (Rana esculenta). *Z vergl. Physiol.* 1967 *55* 299–306.

DAVAL, G J LEVETEAU and P MACLEOD Electroolfactogramme local et discrimination olfactive chez la Grenouille. *J Physiol* (Paris) 1969 *61* Suppl. 2.

DØVING, K. B. and A. J PINCHING, Selective degeneration of neurones in the olfactory bulb following prolonged odour exposure. *Brain Res* 1973 *52* 115–129

DUCHAMP A M F RIVIAL, A HOLLEY and P MACLEOD, Odour discrimination by frog olfactory receptors. *Chem. Senses and Flavor* 1974. *1* 213–233

GESTELAND, R. C., T Y LETTVIN W H PITTS and H ROJAS, Odour specificities of the frog's olfactory receptors. *Olfaction and Taste* Pergamon Press, Oxford, 1963 *1* 19–34

LAND L. J Localized projection of olfactory nerves to rabbit olfactory bulb. *Brain Res* 1973 *6*[illegible] 153–166.

LE GROS CLARK, W E., The projection of the olfactory epithelium on the olfactory bulb in the rabbit *J Neurol Neurosurg Psychiat* 1951 *14* 1 10.

MATHEWS, D F Response Patterns of Single Neurons in the Tortoise Olfactory Epithelium and Olfactory Bulb. *J gen. Physiol.* 1972. *60*. 166–180.

MOZELL, M M and PFAFFMANN, C., The afferent neural processes in odor perception. *Ann. N Y Acad Science* 1954 *58* 96–108

MUSTAPARTA, H Spatial Distribution of Receptor Responses to Stimulation with Different Odours *Acta physiol. scand.* 1971 *82* 154–166.

OTTOSON, D Sustained potentials evoked by olfactory stimulation. *Acta physiol. scand.* 1954. *32*. 384–386

OTTOSON, D Analysis of the electrical activity of the olfactory epithelium. *Acta physiol. scand* 1956 *35* Suppl. 122. 1–83.

OTTOSON, D Studies on slow potentials i the rabbit olfactory bulb and nasal mucosa. *Acta physiol. scand.* 1959 *47* 136–148.

OTTOSON, D The electro-olfactogram. A review of tudies on the receptor potential of the olfactory organ. In *Handbook of Sensory Physiology* Springer Verlag. 1971 *4* 95–131

PINCHING, A. J and K B. DØVING, Selective degeneration in the rat olfactory bulb following exposure to different odours. *Brain Res.* 1974 *82* 195–204

SHARP F R., J S. KAUER and G SHEPHERD Local sites of activityrelated glucose metabolism in rat olfactory bulb during olfactory stimulation. *Brain Res* 1975 *98* 596–600

[illegible] butyrate (3 [illegible] 10^{-4} M) and histamine (0.6 [illegible] 10^{-4} M) to the nutrient solution together with continuous [illegible] of histamine at [illegible] rate of 1 [illegible] 10^{-6} mol min^{-1}. All solutions were adjusted to the same calculated [illegible] as the nutrient solution described above, by altering the NaCl content.

The effect of aspirin (Sigma Chemical Co.) [illegible] as determined by substitution of the nutrient side solution [illegible] a similar one containing approximately 3 mM ASA, during the second period (30–60 min) of the expt. [illegible] exposure to the drug, both the secretory and nutrient side solutions were removed and replaced with [illegible]. The transmucosal flux of ASA was determined from the total salicylate concentration in the secretory [illegible] solution, measured spectrophotometrically at 301 nm (Carl Zeiss PMQ II spectrophotometer) following [illegible] in 5 [illegible] trichloroacetic acid for 30 min at 90°C.

Results

[illegible] of data

[illegible] values for PD, R and I_{sc} during each 30 min period and the mean secretory rate during [illegible] 15 min periods, were obtained for individual expts. The overall mean ± one standard error (S.E.) for each experimental group is presented. Statistical significance was determined using Student's "t" test, by comparing data during the last 4 periods (30–150 min) i.e. during and after administration of ASA, with that from the first 30 min period.

There [illegible] no significant differences ($p > 0.10$) among electrical parameters and secretory rates in any of the 4 control groups during the different time periods. The coefficients of variation were large, but this was due to differences between individual mucosae. In the controls, variation within each expt. was low, reflecting the good stability conditions obtained during the first 3–4 h after isolation of the tissue.

Alkalinization in Necturus antrum

The electrophysiological properties of *Necturus* gastric antrum are shown in Table 1. This tissue developed [illegible] small PD and low I_{sc} compared with the fundic mucosal preparations. In addition, spontaneous alkalinization of the luminal side occurred with a mean secretory rate of 0.38 μeq cm [illegible] h [illegible]. Exposure of the nutrient side to 3 mM ASA for 30 min resulted in a significant decrease in transmucosal PD (0.02 [illegible] $p < 0.05$). When the drug was removed the PD continued to decline and there was also a fall in I_{sc} (p [illegible] 0.01 for both). However there [illegible] as no significant change in R (p [illegible] 0.10), either during or after exposure to the drug. The effect of ASA on spontaneous alkalinization is shown in Fig. 1. When ASA wa[s] present in the nutrient solution, secretion ceased in 4 out of 7 mucosae and was great[ly]

TABLE 1. Electrical properties of control and ASA-treated *Necturus* antral mucosae during spontaneous alkalinization. ASA (3 mM) was applied to the nutrient side during the second period of t[he] expt. (30–60 min). Mean values ± S.E. are given ([illegible] number of expts.).

		0–30 min	30–60 min	60–90 min	90–120 min	120–150 min
Control (n [illegible] 5)	PD (mV)	5.14 ± 0.55	5.27 ± 0.64	5.38 ± 0.72	5.31 ± 0.78	5.17 ± 0.73
	R (Ω cm²)	441 ± 52	436 ± 53	444 ± 53	449 ± 54	471 ± 120
	I_{sc} (μeq cm^{-2} h^{-1})	0.33 ± 0.06	0.39 ± 0.08	0.41 ± 0.09	0.41 ± 0.10	0.39 ± 0.14
			ASA			
ASA-treated (n [illegible] 7)	PD (mV)	6.68 ± 0.58	5.53 ± 0.64	4.33 ± 0.49	3.57 ± 0.32	2.98 ± 0.28
	R (Ω cm²)	427 ± 39	392 ± 27	387 ± 33	411 ± 37	414 ± 40
	I_{sc} (μeq cm^{-2} h^{-1})	0.49 ± 0.05	0.43 ± 0.05	0.35 ± 0.05	0.27 ± 0.04	0.21 ± 0.04

bility which results in a loss of luminal H^+ by back-diffusion into the mucosa (Dave 1967) There is usually a decrease in acid secretion *in vitro* (Flemström and Marsden Kasbekar 1973) although the fact that only net flux is measured may be responsibl some of the conflicting reports concerning the influence of aspirin on H secretion i (Davenport 1964 Lynch, Shaw and Milton 1964).

Recent *in vitro* studies have shown that the surface epithelial cells of the antral m of *Necturus* (Flemström and Sachs 1975) and the fundic mucosa of *Rana temporaria* (I ström 1976), in which acid secretion was inhibited with the histamine H receptor anta Burimamide, produced an alkaline secretion which was sensitive to inhibitors of metabolism and to acetazolamide. The role of an active alkalinization may be to p the antral luminal surface from exposure to a high concentration of H^+ and it migh be one mechanism neutralizing the acid gastric contents. The effect of drugs on the p of alkalinization may further complicate the measurement of gastric acid secretion normally masks the simultaneous but quantitatively smaller alkaline secretion.

It was of interest therefore, to examine the effects of aspirin on the electrical pro and processes of acidification or alkalinization in isolated gastric mucosae from *temporaria* and *Necturus* in order to further study the mechanism by which salicylates gastric secretion. In addition, this approach provides information on the mechani salicylate-induced gastric damage in terms of the relative sensitivity of different sec mucosal cell types to the drug.

Methods

Necturus were purchased from Mogul-Ed, Wisconsin, USA and *Rana temporaria* from Firma Lauingen-Donau, Germany The animals were kept in tap water at 10°C for up to 6 weeks during time they were force-fed with 100–200 g of liver once a week. No food was given during the two d ceding an expt

Animals were killed by section of the spinal cord, the abdomen opened and the stomach remov mucosa was separated from the rest of the stomach wall by blunt dissection in frog-Ringer solutio mounted as a membrane between the two halves of Perspex chamber The exposed mucosal surfa 1 8 cm^2 In the case of *Rana temporaria* only fundic mucosae were used. With *Necturus* separat were performed on mucosae from both the antral and fundic parts of the stomach. The antrum c readily distinguished by its paler appearance and the absence of folds. Both sides of the mucosa were with 20 ml of solution circulated by means of gas lift. The nutrient (serosal) side solution was gas 95% O_2 – 5% CO_2 and the secretory (luminal) side sol tion with 100% O_2, prewashed in $Ba(OH)_2$ to contamination with CO_2. The nutrient solution had the following composition 81.6 mM NaCl, 3 KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.8 mM KH_2PO_4, 17 8 mM $NaHCO_3$, 2.0 mM Glucose, pH A similar but unbuffered solution was used on the secretory side and the pH kept constant at 7 40 b sion of HCl (5 10^{-3} M) or NaOH (15 10^{-3} M) under automatic control from a pH-stat test (Radiometer Copenhagen) The rates of alkalinization or acid secretion were calculated from the a of HCl or NaOH infused.

The transmucosal potential difference (PD) was measured via 2 matched calomel electrodes and re with a high-input impedance voltmeter Short-circuit current (I_{sc}) was applied from an external sou Ag-AgCl electrodes separated from the circulating solution by thick cellophane membranes. The was adjusted manually and calculated from the voltage drop over a precision resistor The electrica ance (R) was determined from the change i potential immediately following passage of a fixed (30 μA cm^{-2}) through the tissue in either direction.

Electrical parameters and secretory rate were measured every 5 min duri g 5 consecutive 30 min p 4 experimental groups, based on secretory differences, were used. In *Necturus* mucosae, alkalin (antrum) and acid secretion (fundus) were spontaneous. Alkalinization in *Rana temporaria* fund induced by addition of Metiamide (10^{-3} M) to the trient side solution, and acid secretion by addi

ble II Electrical properties of control and ASA-treated *Rana temporaria* fundic mucosae during Metiamide-induced alkalinization. ASA (3 mM) was applied to the nutrient side during the second period of the expt. (30–60 min). Mean values ±S.E. are given (n = number of expts.).

		0–30 min	30–60 min	60–90 min	90–120 min	120–150 min
ntrol (5)	PD (mV)	14 60±2.83	14 15±2.74	13.67±2.60	13.34±2.59	13 08±2.53
	R (Ω cm²)	536±104	523±89	528±91	333±97	532±101
	I_{sc} (μeq cm⁻² h⁻¹)	0.97±0.15	0.94±0.16	0.92±0.16	0.90±0.16	0 88±0.15
			ASA			
A-treated (8)	PD (mV)	15.39±2.71	10 69±2.54	11.98±2 48	12.27±2.29	11.93±2.22
	R (Ω cm²)	334±83	365±82	347±84	345±86	345±89
	I_{sc} (μeq cm⁻² h⁻¹)	1 06±0.40	1.22±0.32	1.42±0 32	1 44+0.30	1 40±0.29

lkalinization of the secretory side following the disappearance of spontaneous acid secretion lenström 1976).

The electrical properties and secretory rates in control and ASA-treated fundic mucosae om *Rana temporaria* in which alkalinization was induced with the histamine H receptor tagonist Metiamide (10 M) are shown in Table II and Fig. 2. The results reported are om mucosae in which a steady rate of alkalinization was recorded within 2 h (mean = 5 min), which occurred in 13 out of a total of 21 preparations. Metiamide-treated mucosae d a lower PD and I_{sc} and a higher R than acid secreting mucosae from the same species ee Table IV). The rate of alkaline secretion in the control group (~0.46 μeq cm h⁻¹) was milar to that in the *Necturus* antrum (~0.38 μeq cm⁻² h⁻¹). Addition of ASA (3 mM) sulted in a significant fall in PD and I_{sc} (0 02 $p < 0.05$ for both) but no change in R. After moval of the drug, the electrical parameters were not significantly different ($p > 0.10$) an before administration. There was reversal in secretory response during the period

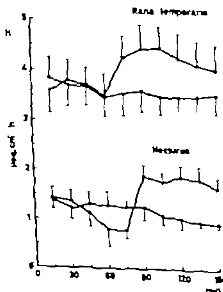


Fig. 3 Rates of spontaneous acid secretion (*Necturus*) and histamine-stimulated acid secretion (*Rana temporaria*) in control and ASA-treated fundic mucosae. *ASA* (3 mM) was applied to the nutrient side of the tissue for 30 min during the second period of the expt. (30–60 min). Mean values ±S.E. are given. Control (open circles, 4 for *Necturus* and 5 for *Rana temporaria*) and ASA-treated (filled circles, 6 for *Necturus* and 7 for *Rana temporaria*).

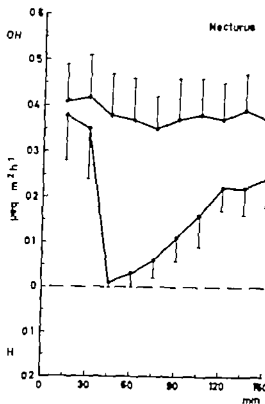


Fig. 1

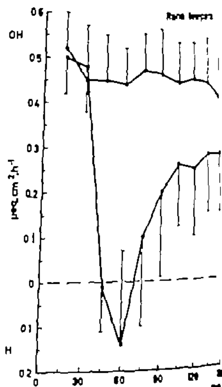


Fig. 2

Fig. 1 Rates of spontaneous alkalinization in control and ASA treated *Necturus* antral mucosa. ASA (3 mM) was applied t the nutrient side of the tissue for 30 min during the second period of the experiment (30–60 min). Mean values ±S.E. are given. Control (open circles, n = 5) and ASA treated (filled circles, n = 7).

Fig. 2. Rates of Metiamide-induced alkalinization in control and ASA treated *Rana temporaria* fundic mucosa. ASA (3 mM) was applied to the nutrient side of the tissue for 30 min during the second period of the expt. (30–60 min). Mean values ±S.E. are given. Control (open circles, n = 5) and ASA-treated (filled circles, n = 8).

reduced in the remainder ($p < 0.001$) There was a partial recovery in response following wash-out of the drug but the rate was less than before administration ($p < 0.01$). When 3 mM ASA was applied to the secretory side (n = 2), alkalinization during the period 60–150 min was totally abolished. In this case, however it was not possible to titrate secretion while the drug was present during which time the pH of the solution rose by 0.7 units. The antral mucosa has no acid secretory capacity as evidenced by the absence of acid secretion after challenge with histamine (10^{-4} M) and in this tissue, unlike *Rana temporaria* fundus (see Fig. 2), cessation of alkalinization was not associated with an onset of acid secretion.

Alkalinization in Rana temporaria fundus

The gastric mucosa from most amphibian species *in vitro* secretes acid spontaneously (Hogben 1965) due largely to stimulation of acid secreting cells by endogenous histamine released from mucosal stores (Kasbekar 1967 Rangachari 1975) Addition of the histamine antagonist Burimamide to the nutrient side of isolated fundic mucosae is associated with a

Transmucosal flux of ASA in *Necturus* and *Rana temporaria* isolated gastric mucosae. The nutrient side of the tissue was exposed to ASA for 30 min and the unidirectional flux calculated from the concentration in the secretory side solution. Drug concentration as determined as total salicylate. Mean values ± S.E. are given (= number of expts).

	Secretory	Initial concentration on nutrient side (mM)	Final concentration on secretory side (mM)	Flux (μmol cm⁻² min⁻¹)
(n 7)	Alkalinization	3.02 ± 0.14	0.12 ± 0.02	19 ± 0.37
mporaria (n 8)	Alkalinization	3.09 ± 0.13	0.19 ± 0.04	3.57 ± 0.49
(6)	Acidification	3.00 ± 0.06	0.20 ± 0.01	3.73 ± 0.25
mporaria (n 6)	Acidification	2.84 ± 0.13	0 4 ± 0.01	4.38 ± 0.21

corded by Flemström (1971). In this tissue, PD and I_{sc} were higher and R lower p 0.02 for all) than in the same species during Metiamide-induced alkalinization or eturus fundus during spontaneous acid secretion. There was no significant change in f the electrical parameters (p 0.10) following exposure of the mucosa to ASA (Table The PD tended to fall by 3–4 mV throughout the course of the expt., but this also oc d in the control group. The mean rate of acid secretion was 3.6 μeq cm⁻² h⁻¹ and ined unchanged in the presence of ASA (Fig. 3). On removal of the drug the rate of secretion increased, as also found in the spontaneously secreting *Necturus* fundus, but ffect was only of low significance (0.05 p 0.10).

mucosal flux of ASA

e present expts. ASA (pK_a 3.6) was added to the buffered nutrient side solution at 7.2 and was therefore present in the ionized form. The flux of ASA from the nutrient to etory side of the membrane was determined as total salicylate and is shown in Table V ansmucosal flux was greatest in the acid secreting *Rana temporaria* fundus (4.38 μmol ⁻² min⁻¹), where the secretory side concentration after 30 min amounted to 8.45% of the inistered dose. This tissue also had the lowest recorded mucosal resistance. However cylate flux was not significantly different (p 0.05) than that in the same tissue during linization or in *Necturus* fundus during spontaneous acid secretion. The lowest flux urred in the *Necturus* antrum (2.19 μmol cm⁻² h⁻¹) and was significantly less (0.02 < p < 5) than in all other tissues.

Discussion

stric physiologists have been mainly concerned with the acid secreting fundic mucosa and ly recently have investigations been directed at the antral region of the stomach. The ectrophysiological properties of *Necturus* antrum were characterised by Flemström and chs (1975), who reported that this tissue generated a low PD (4–10 mV) and I_{sc} (~0.41 μeq ⁻² h⁻¹) dependant on mucosal to serosal Na transport, but had a relatively high resist

TABLE III Electrical properties of control and ASA treated *Necturus* fundic mucosa during spontaneous acid secretion. ASA (3 mM) was applied to the nutrient side during the second period of the expt. (30–60 min). Mean values ±S.E. are given (n = number of expts.).

		0–30 min	30–60 min (ASA)	60–90 min	90–120 min	120–150
Control (n = 4)	PD (mV)	16.02±1.90	16.33±1.44	15.85±1.29	15.30±1.08	15.18 [illegible]
	R (Ω cm²)	500±113	480±102	484±88	506±95	516±[illegible]
	I_{sc} (µeq cm⁻² h⁻¹)	1.26±0.12	1.34±0.13	1.25±0.12	1.15±0.08	1.13±[illegible]
ASA treated (n = 6)	PD (mV)	15.83±1.53	14.04±2.26	17.08±1.97	15.54±2.08	15.63±[illegible]
	R (Ω cm²)	470±46	563±77	444±77	490±78	[illegible]
	I_{sc} (µeq cm⁻² h⁻¹)	0.84±0.16	0.72±0.19	0.86±0.26	0.87±0.26	0.83 [illegible]

in which ASA was present in the nutrient side solution and although an alkaline secr[illegible] reappeared when the drug was removed, the rate was less than before administration (0[illegible] $p < 0.05$)

Acidification in Necturus fundus

In contrast with mucosa from the antral part of the stomach, the fundic mucosa of Ne[illegible] secreted acid spontaneously (~1.2 µeq cm⁻² h⁻¹) and had a considerably higher PD [illegible] greater I_{sc}. However the value of R in the two tissues was similar. The effect of nu[illegible] side ASA (3 mM) on the rate of acid secretion is shown in Fig. 3. There was a slight inhi[illegible] of secretion during exposure to the drug and in the first 15 min after its removal (0.05 [illegible] 0.1) following which the rate of secretion increased to a higher level than before [illegible] ($0.02 < p < 0.05$). The decrease in acid secretion was accompanied by a fall in PD a[illegible] and an increase in R (Table III). There was a small rise in PD and I_{sc} together with a [illegible] R during the subsequent increase in rate of secretion. However the changes in elec[illegible] activity were not significant ($p > 0.10$). There was no change in the rate of acid sec[illegible] following secretory side application of 3 mM ASA ($n = 2$).

Acidification in Rana temporaria fundus

Addition of butyrate and continuous infusion of histamine into the nutrient solution [illegible] used to obtain good long-term stability of acid secretion in the isolated fundic muc[illegible]

TABLE IV Electrical properties of control and ASA treated *Rana temporaria* fundic mucosa [illegible] histamine-stimulated acid secretion. ASA (3 mM) was applied to the nutrient side duri[illegible] second period of the expt. (30–60 min). Mean values ±S.E. are given (n = number of ex[illegible]

		0–30 min	30–60 min (ASA)	60–90 min	90–120 min	120–150
Control (n = 5)	PD (mV)	25.74±3.52	[illegible]4.71±3.58	23.65±3.51	[illegible]57±3.38	21.87±[illegible]
	R (Ω cm²)	46[illegible]±35	25[illegible]±54	275±60	267±53	[illegible]
	I_{sc} (µeq cm⁻² h⁻¹)	2.43±0.41	2.40±0.41	2.34±0.42	2.28±0.40	2.[illegible]6 [illegible]
ASA treated (n = 7)	PD (mV)	26.39±3.75	[illegible]3.65±3.26	23.05±3.72	22.20±3.77	[illegible]2.73±[illegible]
	R (Ω cm²)	200±26	240±19	215±[illegible]7	[illegible]22±[illegible]4	217±[illegible]
	I_{sc} (µeq cm⁻² h⁻¹)	3.19±0.39	[illegible]91±0.38	3.00±0.47	2.97±0.49	3.06±[illegible]

unionised form (Flemström and Marsden 1973). Although the drug permeated the gastric mucosa, the pK_a value precludes it from contributing to the titratable acidity of the secretory solution. The unchanged rate of acid secretion in *Rana temporaria* fundus in the presence of ASA, which markedly reduced alkalinization in the same tissue, is a further indication that the drug caused a simultaneous inhibition of acid secretion.

The rate of acid secretion increased in both species following removal of ASA. This effect was only of low significance in the *Rana temporaria* and was probably due to the fact that any increase is superimposed on the already high rate of histamine-stimulated secretion. The poor recovery of alkaline secretion in both the antrum and fundus, combined with the fact that ASA inhibited acid output in *Necturus* fundus, makes it unlikely that the rise in acid secretion after ASA was due to actual stimulation. Furthermore, the post-ASA secretory profiles strongly suggest that under the present *in vitro* conditions at least, measured secretory response is a reflection of the balance between acid and alkali output.

The rate of passage of drugs across biological membranes decreases following administration in the ionized form, and the transmucosal flux of ASA in these expts. was low. The smallest flux occurred in the antrum, where the secretory side concentration after 30 min was only 4% of the applied dose. However the mucosal flux was not related to the secretory response, since in this tissue there was an almost total inhibition of alkalinization. The drug concentration in the mucosa, and in particular within the secretory cells, is of more relevance. Although not measured in the present study, indirect measurement based on the difference between the concentration administered and the sum of that recovered in the secretory and nutrient solutions, indicated that mucosal concentration was greatest in the antrum.

The origin of the alkaline secretion from the gastric mucosa is unknown but a number of observations suggest that it is the surface epithelial cell. These cells contain most of the gastric mucosal carbonic anhydrase (Vollrath 1959) and the gastric antrum is composed mainly of this type of cell. Also, ASA was a more effective inhibitor of alkalinization when applied to the luminal side of the mucosa than from the serosal side. The weak base aminopyrine was found to accumulate in parietal cells (Berglindh, Helander and Öbrink 1976) suggesting that the cytoplasm of these cells is basic. Conversely it would be expected that ASA could accumulate preferentially in the surface epithelial cells and this may be responsible for the greater sensitivity of alkalinization compared with acid secretion to the inhibitory actions of the drug. The present results support the electron microscopical observations of Hingson and Ito (1971), who reported that the surface epithelial cells of mouse gastric mucosa were particularly susceptible to the damaging effects of ASA, but concluded that the action of the drug was not directed at secretory function in particular. However the drug was administered in a higher dose than in the present study and from the luminal side in the unionized form. Under these conditions it is to be expected that a considerably higher intracellular concentration would be achieved and that general cellular damage would result. The present study suggests that a much lower concentration is required in order to affect the secretory function of the mucosa and furthermore that this function is extremely sensitive to ASA.

In the amphibian gastric mucosa *in vitro* alkalinization is considerably more sensitive than acid secretion to the effects of ASA. Inhibition of alkalinization is associated with an

ance. There was also an active alkalinization of the luminal surface. Results from the present study are in good agreement with their data in respect to electrical parameters. In addition the tissue was found to secrete alkali spontaneously at a steady rate which amounted to 13% of the rate of spontaneous acid secretion by the fundic mucosa from the same species.

Quantitation of conductance pathways in the antrum have shown that it is a "leaky" epithelium in which transport is due mainly to the extracellular passage of ions (Spenney et al 1975) Alkalinization may be of importance, therefore, in protecting the mucosa from the large H^+ gradient generated by the adjacent fundus, which is a "tight" epithelium (Spenney Shoemaker and Sachs 1974) The high shunt conductance may also be of relevance in the transmucosal flux of weak electrolytes such as ASA, since there is evidence that drugs in the ionized form may permeate biological membranes via a paracellular route (Chang et al 1970 Wright and Pietras 1973).

The gastric antrum has no acid secretory capacity as gauged by the absence of an effect with histamine. Furthermore, the abolition of alkalinization in the antrum following ASA administration was not followed by the appearance of acid secretion. The assessment of fundic secretion, however is complicated by the fact that this tissue has the ability to secrete both acid and alkali. Since it is not possible to titrate acid and alkali output simultaneously the measured (net) secretion is only a reflection of the dominance of one or other of these processes. The spontaneous secretion in *Necturus* fundus was always acid, although the tissue does secrete alkali when acid secretion is inhibited with Burimamide (Flemström 1976 In *Rana temporaria* fundus, spontaneous alkalinization occurred in 4 out of 13 mucosae from the start of the expt. and the remainder had an alkalinization revealed following inhibition of acid secretion with Metiamide This drug was used in preference to Burimamide which induces alkalinization in only 60% of cases (Flemström, personal communication

Alkalinization is an active process which is inhibited by antimetabolites and acetazolamide (Flemström 1976) Acetazolamide has been demonstrated to cause gastric mucosal erosion in the dog (Werther Hollander and Altamirano 1965) and may act by reducing the formation of bicarbonate and consequently the degree of neutralization of the gastric content In the present study ASA, which is well known to cause gastric mucosal damage, produced a marked inhibition of both spontaneous and Metiamide-induced alkalinization. This observation itself makes it unlikely that alkalinization is due to the passive permeation of bicarbonate from the nutrient solution since administration of ASA was associated with [illegible] increase in mucosal conductance. In addition Metiamide which was used to initiate alkalinization in the frog, also caused an increase in conductance. An increase in the luminal bicarbonate concentration occurred *in vivo* following oral administration of 20 mmol ASA the unionized form (Davenport 1966) and when the interstitial pressure of the gastric mucosa was increased (Altamirano Requena and Pérez 1975) However in these cases it is like that there is an ultrafiltration of bicarbonate resulting from an increase in the permeability of the paracellular pathways of the mucosa.

The decrease in alkalinization in *Rana temporaria* fundus could conceivably result from stimulation of acid secretion by ASA This is unlikely since the drug inhibited acid secretion during the period of administration in *Necturus* fundus and has also been demonstrated to cause inhibition of secretion in *Rana temporaria* fundus when applied to the luminal side

GROSSMAN, M. I., K. K. MATSUMOTO and R. J. LICHTER, Fecal blood loss produced by oral and intravenous administration of various salicylates. *Gastroenterology* 1961 *40*: 383–388.

HINGSON, D. J. and S. ITO, Effect of aspirin and related compounds on the fine structure of mouse gastric mucosa. *Gastroenterology* 1971 *61*: 156–177.

HOGBEN, C. A. M. Introduction: the natural history of the isolated bullfrog mucosa. *Fed. Proc.* 1965 *24*: 1353–1359.

KASBEKAR, D. K., Studies of resting isolated frog gastric mucosa. *Proc. Soc. exp. Biol.* (N.Y.) 1967 *125*: 267–271.

KASBEKAR, D. K. Effects of salicylate and related compounds on gastric HCl secretion. *Amer. J. Physiol.* 1973. *225*: 521–527.

LYNCH, A., H. SHAW and G. W. MILTON, Effect of aspirin on gastric secretion. *Gut* 1964. *5*: 230–236.

RANGACHARI, P. K., Histamine release by gastric stimulants. *Nature* (Lond.) 1975. *253*: 53–55.

SACHS, G., B. I. HIRSCHOWITZ and R. L. SHOEMAKER, Microelectrode studies of aspirin damage to gastric mucosa. *Gastroenterology* 1972. *62*: 804.

SMITH, B. M., J. J. SKILLMAN, B. G. EDWARDS and W. SILEN, Permeability of the human gastric mucosa: alteration by acetylsalicylic acid and ethanol. *New Engl. J. Med.* 1971 *285*: 716–721.

SPENNEY, J. G., R. L. SHOEMAKER and G. SACHS, Microelectrode studies of fundic gastric mucosa: cellular coupling and shunt conductance. *J. Membrane Biol.* 1974 *19*: 105–128.

SPENNEY, J. G., G. FLEMSTRÖM, R. L. SHOEMAKER and G. SACHS, Quantitation of conductance pathways in antral gastric mucosa. *J. gen. Physiol.* 1975. *65*: 645–662.

THORSEN, W. B., D. WESTERN, Y. TANAKA and J. P. MORRISSEY. Aspirin injury to the gastric mucosa. *Arch. intern. Med.* 1968 *121*: 499–506.

VOLLRATH, L., Über Entwicklung und Funktion der Belegzellen der Magendrüsen. *Z. Zellforsch.* 1959 *50*: 36–60.

WERTHER, J. L., F. HOLLANDER and M. ALTAMIRANO, Effect of acetazolamide on gastric mucosa in canine vivo-vitro preparations. *Amer. J. Physiol.* 1965. *209*: 127–133.

WRIGHT, E. M. and R. J. PIETRAS, Routes of nonelectrolyte permeation across epithelial membranes. *J. Membrane Biol.* 1974 *17*: 293–312.

inability of the mucosa to withstand a low intraluminal pH (Flemström 1976). The appar[illegible] increase in acid secretion is probably due to a reduction in the degree of neutralisation [illegible] the lumen. This effect, combined with the increase in mucosal permeability may be re[illegible]sponsible for the gastric damage associated with ASA administration which occurs [illegible] particularly in the antral region of the stomach which is a common site of ulceration [illegible]

Thus in addition to inhibition of parietal secretion *per se* and the loss of H by bac[illegible] diffusion an important consideration in the assessment of gastric secretion should be [illegible] relative contributions of acid and alkaline output. In fact the effect of drugs on the ph[illegible]nomena of neutralisation may be more significant than an effect on back-diffusion, [illegible] mucosal conductance was unaffected or marginally increased after ASA and quickly [illegible]covered on removal of the drug, whereas secretory response showed only a slow rate of [illegible]covery

This work was conducted during the tenure of a Royal Society fellowship (European Programme) [illegible] study was supported by grants from the Royal Society the Swedish Medical Research Council [illegible] and the Medical F culty of the University of Uppsala which are gratefully acknowledged. The author [illegible] to thank Dr G Flemström for critical comments during the preparation of the manuscript. [illegible] was a kind gift from Dr M E. Parsons, Smith Kline & French Lab Herts., U.K.

References

ALTAMIRANO, M M REQUENA and T C. PÉREZ, Interstitial fluid pressure in canine gastric mucosa. *A[illegible] J Physiol* 1975 *229* 1414–1420.

ANDERSON, K. W A study of the gastric lesions induced by aspirin in laboratory animals *Arch. int. P[illegible]macodyn.* 1964 *152.* 379–391

BERGLINDH, T H. F HELANDER and K. J ÖBRINK, Effects of secretagogues on oxygen consump[illegible] aminopyrine accumulation and morphology in isolated gastric glands. *Acta physiol. scand.* 197[illegible] 401–414

CHUNG, R. S. K., P T SUM, H GOLDMAN M FIELD and W SILEN, Effects of chelation of calcium o[illegible] gastric mucosal barrier *Gastroenterology* 1966. *59* 200–207

CROFT D N The action of analgesic substances on the gastric mucosa. *J Pharm. Pharmacol.* 196[illegible] 354–361

DAVENPORT H. W Gastric mucosal injury by fatty nd acetylsalicylic acids. *Gastroenterology* 196[illegible] 245–253

DAVENPORT H W Fluid produced by the gastric mucosa during damage by acetic and salicylic [illegible] *Gastroenterology* 1966. *50* 487–499

DAVENPORT H W Salicylate damage to the gastric mucosal barrier *New Engl J Med.* 1967 *276* [illegible] 1312.

DAVENPORT H W Gastric mucosal haemorrhage in dogs, effects of acid aspirin and alcohol *Ga[illegible]enterology* 1969 *56* 439–449

FLEMSTRÖM, G Na transport and impedance properties of the isolated frog gastric mucosa at di[illegible] O_2 tensions. *Biochim. biophys. A t* (Amst.) 1971 *225* 35–45

FLEMSTRÖM, G., Properties of isolated gastric antral and non-acid secreting fundic mucosa In *G[illegible] Hydrogen Ion Secretion*, ed. D K. Kasbekar G Sachs nd W S. Rehm. Marcel Dekker Inc., New [illegible] 1976. p. 102 116.

FLEMSTRÖM, G Stimulation of gastric alkaline secretio by carbacholine and db-cGMP *S mposiu[illegible] the Biochemistry of Membrane Transport* Zürich, Switzerland. *FEBS Letters.* 1976. p. 49

FLEMSTRÖM, G and N. V B MARSDEN, Dextran permeability electrical properties and H -secret[illegible] isolated frog gastric mucosa after acetylsalicylic acid *Gastroenterology* 1973. *64* [illegible]78–284

FLEMSTRÖM, G. and G SACHS, Ion transport by amph bian antrum i vitro *Amer J Physiol.* 1975 1188–1198

FRENNING, B., The disappearance of acetylsalicylic acid (ASA) aqueous solution from the cat sto[illegible] and its influence on the transmucosal ion transport in the innervated non-secreting stomach. *Acta ph[illegible] scand.* 1971 *83* 235–246.

cell bodies in the geniculate ganglion outside the CNS while the efferent fibres have cell bodies in the CNS (van Buskirk 1945 Foley and DuBois 1943 Foley 1945, Villiger Boudreau et al. 1971). Therefore, section of the facial root between the CNS and the [illegible]ion ought to cause degeneration of the efferent fibres while the sensory remain. In the [illegible]nt study this section has been made in rats. The paper will give a first report of some of [illegible]sults obtained.

Methods

[illegible]periments to be described were done on adult male Sprague-Dawley rats weighing 500 ± 50 g. The [illegible]y was performed under [illegible] combination of 2 anesthetic drugs. The anesthesia has recently been de-[illegible]d (Hellekant and Gopal 1975) and will only be reported briefly here. The two drugs used were Hyp-[illegible] and Mebumal[?] The proportion in [illegible]hich the drugs were used was adapted to the experimental [illegible]ments. Less Mebumal than Hypnorm was used [illegible]hen the facial root [illegible]as cut and more during the [illegible]al expt [illegible]hen the tongue was perfused

[illegible] surgery for cutting the facial root was as follows. The skin on the head [illegible]as divided by [illegible] sagittal cut [illegible]bregma rostrally to the occipital crest. The tissue overlaying the interparietal bone was divided with [illegible]verse cut and the bone exposed. A 3 [illegible] 4 mm piece of the bone from the right lateral border to about [illegible] on the other side of the midline was removed. The meninges were divided to expose the cerebellum. [illegible]erebellum was gently retracted from the right side of the cranial cavity until the root of the facial nerve [illegible]as exposed. The root was then transected. Care was taken not to damage the VIII nerve which enters [illegible]ventral and somewhat posterior to the facial nerve. The wound was then closed and sutured in two [illegible]s with silk. The piece of bone was not returned. After surgery 10 ml Andoncol glucose[?] and 0.2 ml [illegible]mycin [illegible] were given [illegible]c. Once the technique was developed only one out of 30 rats died as [illegible] direct [illegible]lt of surgery

[illegible]ter intervals ranging from [illegible] week to 3 months the chorda tympani proper nerve outside the tympanic [illegible] was dissected. The nerve was cut and put on electrodes to allow recording from and electrical stimula-[illegible] of its peripheral part. The vascular resistance of the tongue or the temperature of its surface or both [illegible] recorded. The technique for studying the vascular resistance of the tongue was described earlier [illegible]llekant 1971 b) but, as [illegible] has been slightly modified, it will be repeated here.

[illegible]he tongue was unilaterally perfused at [illegible] constant flow rate [illegible]hile the perfusion pressure was recorded. [illegible] perfusion loop consisted of 2 polythene tubes connected via two 3-way cocks with an intermediate [illegible]cone rubber tube [illegible]hich was placed in [illegible] peristaltic pump, Holter RD 074. The loop had [illegible] volume of [illegible]ml. The cocks made it possible to direct the flow either to the tongue or to [illegible] femoral vein and to flush [illegible] system [illegible]ith Ringer-Heparin[?] solution. During the perfusion blood was taken from the right femoral [illegible]ery forced through the loop by the pump and ejected through the right external maxillary artery into [illegible] external carotid artery from [illegible]hich the lingual artery branches. Thus the tongue could be supplied with [illegible]od in the normal way until perfusion started. Then the pump was started and the external carotid artery [illegible]s clamped between the heart and the branching of the lingual artery. Other arterial branches were per-[illegible]nently occluded. That was also the case [illegible]ith the branch [illegible]hich in about one-third of the rats is given off [illegible] the lingual artery close to its origin and [illegible]hich supplies the surrounding tissue and not the corpus of [illegible] tongue. The perfusion pressure was measured in the right posterior auricular artery close to its orifice [illegible] the external carotid artery. The contralateral blood supply to the tongue was not touched. This seemed [illegible] be justified because each half of the tongue is largely supplied with blood from the ipsilateral lingual [illegible]tery (Hellekant 1971 [illegible])

The temperature of the surface of the tongue was measured by [illegible] thermocouple attached to the tongue [illegible]uch [illegible]ay that the temperature difference between the right and left dorsal sides of the tongue was [illegible]corded. The thermocouple [illegible]as connected to [illegible] DC amplifier [illegible]hose output was recorded. The systemic [illegible]lood pressure [illegible]as recorded in the right femoral artery. Electrical stimulation was delivered from [illegible] Grass [illegible]imulator (S88). The stimuli were pulses with [illegible] duration of 2 ms and [illegible] current of 10–20 μA. Their frequency [illegible]aried from 1 [illegible] 100 Hz.

Results

The records show[illegible] in Fig. 1 were obtained during perfusion of the tongue in a normal rat. The top trace records the blood pressure to the tongue. The second trace shows the systemic

Acta physiol. scand. 1977 99 292–299

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Vasodilator Fibres to the Tongue in the Chorda Tympani Proper Nerve

By

GÖRAN HELLEKANT

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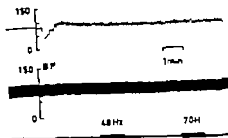
Abstract

HELLEKANT G *Vasodilator fibres to the tongue in the chorda tympani proper nerve* Acta physiol scand. 1977 99 292–299

Electrical stimulation of the chorda tympani proper nerve (CT) causes vasod latation in the constant flow perfused tongue. It has been suggested as an explanation for this, that the vasod latation is the result of a tidromic impulses in sensory fibres of the CT However after degeneration of the efferent fibres in the CT in the rat it was found that electrical stimulation of the CT caused no vasodilatation. Since, by all criteria available, the sensory fibres remained, the result indicates that antidromic impulses could not be the cause of the vasodilatation observed in tongues with intact nerve supply Further since there are no glands in the part of the tongue innervated by the CT and the circulation of the large salivary glands is separate from the tongue in the rat, it is concluded that the vasodilatation was not a secondary effect of glandular activity but was caused by vasodilator fibres in the CT

Ever since Claude Bernard (1858) described how electrical stimulation of the chorda tympani proper nerve, CT produced vasodilatation in the submandibular salivary gland, the question has puzzled physiologists whether it, in addition to its salivary and afferent fibres, contains specific vasodilator fibres. Though the evidence for specific vasodilator fibres in the nerve to the submandibular salivary glands is growing (Gautvik 1970) it has not been possible to prove conclusively that this vasodilatation is not a secondary effect of the secretion caused. A similar difficulty exists with regard to the vasodilatation in the tongue. Though there are no glands in the anterior part of the tongue, it can always be claimed that the vasodilatation observed was the result of antidromic impulses in the sensory fibres of the CT nerve (Eriksson and Uvnäs 1952, Eccles and Wallis 1974). We have earlier observed and described changes in the efferent activity of the CT which indicate that it contains vascular fibres (Hellekant 1972). We have also recorded changes of the vascular resistance of the tongue to stimulation of this nerve (Hellekant 1970) but we have not been able to rule out the argument presented above.

However we have now developed a technique which seems to cause degeneration of all efferent fibres in the nerve without destroying its sensory portion. The sensory fibres have



...he vascular resistance, blood pressure and ...e periods were recorded as in the previous ...t in an animal whose right facial root had . The record shows that stimulation of the ...s case did not elicit decrease of vascular ...of the tongue though stronger stimulation , 40 and 70 Hz.

was in contrast to the results obtained in rats which had their facial root cut. Fig. 3 records from an expt. in such a rat. The same procedure was applied as in Fig. 1 but ...ulus rates were higher 40 and 70 Hz. It is seen in Fig. 3 that in spite of this strong ...tion no decrease of vascular resistance was recorded.

...e expts. were repeated in 15 rats of which every second animal had its facial root cut. ...sults were similar. However in 4 of the animals with cut facial root a slight decrease ...fusion pressure was traced at stimulus rates above 40 Hz. Quantification of these ...was difficult, but because any effects along these lines after the facial root was cut ...d important, it was tried. The decrease observed in one of these animals was estimated ...at maximum 10% in the other 5%. It is evident that these effects were small compared ...se recorded in a normal animal, Fig. 1 and Fig. 2. In fact the decreases were difficult ...cover. Similar small effects could be observed in these animals during strong stimula-... (40 Hz) of their lingual proper nerves.

...conclusion, it seems that there was a basic difference between the normal animals ...those with cut facial nerves. Only in animals in which the efferent fibres in their chorda ...ani nerve remained could a substantial decrease of vascular resistance of the tongue be ...ed. The effects in the "cut" animals were only observed during strong unphysiological ...ulation (40 Hz). It may be of interest that they could also be elicited by stimulation of ...r lingual proper nerves too.

...uring stimulation in unperfused normal rats it was observed that the tongue on the ...ulated side darkened indicating hyperemia on that side. In the unperfused normal rat ...he blood to the tongue was not cooled by the perfusion loop, it seemed reasonable that ...temperature of the tongue should change during stimulation, as the result of the hyper...a. To test this, thermocouple was attached to the tongue, in such a way that a possible ...ference in temperature between the right and left sides could be recorded. The method ...s described under Methods (p. 293). It was then observed that the temperature increased ...the side where the CT nerve was stimulated. This was not observed after the facial root ...d been cut. Further in 12 rats both vascular resistance and tongue temperature were ...corded. Then we recorded the vascular resistance by perfusing during the stimulation, ...itched then to normal circulation and repeated the stimulation, while we recorded the ...mperature difference between the right and the left sides of the tongue. It was then observed ...at an increase of temperature of the tongue never occurred as result of chorda tympani ...mulation if a decreased vascular resistance could not be recorded.

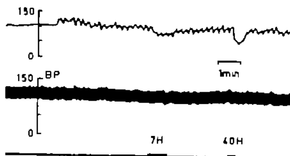


Fig. 1 In the top trace the vascular resistance of the tongue was recorded during perfusion in a normal rat. The middle trace records the systemic arterial blood pressure and the lower trace is a signal indicating two periods of electrical stimulation of the right CT with pulses at 7 and 40 Hz, 20 μA.

blood pressure in one femoral artery The bottom trace is a signal indicating the periods of electrical stimulation of the peripheral part of the chorda tympani proper nerve (CT). The perfusion pump was first started Then the blood supply from the heart was stopped by occlusion of the common carotid artery between the heart and the branching of the lingual artery so that the tongue was supplied with blood from the pump The output of the pump was adjusted to give a flow to the tongue which judged by the blood pressure, was approximately the same as before the occlusion. Then during the constant perfusion the CT was stimulated During the first stimulation the pulses were delivered at 7 Hz and during the second at 40 Hz. The current was 20 μA The record shows that the first stimulation caused moderate vasodilatation while the second gave a strong dilatation in the tongue. The duration and shape of decrease in vascular resistance that was recorded during the stimulation may be of interest Periods of stimulation larger than 1 min were not tried in this series of experiments. The records indicate that the vasodilatation reached a plateau and remained there during stronger stimulation while a slight recovery was observed during less strong stimulation.

Fig. 2 presents in diagrammatic form several observations on the relationship between the changes of the vascular resistance observed and the frequency of stimulation applied during constant-flow perfusion of the tongue in normal animals. The average resistance before each stimulation was considered as 100 Then the change observed during the stimulation was measured and expressed as a percentage of the value before stimulation. In Fig. 2 values from 8 rats are included, but only the stimulus frequency of 40 Hz was applied in all the rats. Otherwise each point comprises data from 3–4 rats. The bars give the SD The diagram is not intended as an exact quantitative description of the stimulus/effect relationship. However certain conclusions can be drawn from the diagram. Thus it seems that larger decreases in vascular resistance than 50% could not be elicited by CT stimulation. Further it suggests that even a stimulus frequency of 1 Hz was able to affect the vascular resistance of the tongue. Finally it shows that the largest vascular changes occurred at changes in the low stimulus frequency range (1–4 Hz) and that maximum effects were obtained at < 20 Hz.

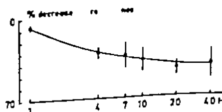


Fig. 2. The decrease of vascular resistance in the tongue of eight normal rats has been plotted against the stimulus frequencies applied to the CT nerves. The bars illustrate the SD The diagram shows that the largest changes are elicited by changes in the lowest frequency range and that a plateau was reached between 10–20 Hz, after which further increase of stimulus frequency did not cause more vasodilatation.

r modalities and efferent fibres to salivary glands and perhaps to other structures
es also (Hellekant 1971). A number of anatomists have investigated the facial
y and DuBois 1943, Beskirk 1945 Foley 1945 Villiger 1964, Boudreau *et al*
nformation available suggests that the sensory fibres of the CT have their cell
in the geniculate ganglion. In the rat this ganglion is a small elongated structure
il nerve (Hellekant, own observations). Its cell bodies do not extend centrally to
where the facial root was cut in the expts. here. Further the cell bodies of
fibres are all in the CNS. Thus it seems that section at the point described here
: fibres in the CT into two groups, a sensory one which still was connected with
ies and a motor group which was disconnected from its cell bodies. According to
wn observation, first described by Waller (1852), this ought to cause degeneration
or fibres in the CT while the sensory fibres will remain. This seemed to be true, here
CT on the cut side contains about 25% fewer myelinated fibres and about 50%
yelinated fibres than the normal side 18-20 days after section of the facial root
and Hellekant, to be published). The sensory fibres seem vital both from morpho-
d physiological points of view because a good response to taste stimulation of the
is recorded from the peripheral part of the CT No activity was observed in the
irt (Hellekant, own observations) which indicates that the efferent fibres had
ed. In the normal rat nerve impulses can be recorded from the central part, as has
ribed earlier (Hellekant 1971 a, b, c, 1973 a, b, 1974). Thus it seems that the tech-
d caused degeneration of the efferent fibres in the CT while the sensory remained
ioned. In spite of this, as has been described, electrical stimulation of the CT nerve
o significant dilatation of the blood vessels of the tongue. This suggests strongly
fromic impulses in the CT nerv cannot be used as an explanation for this dilatation.
er there are two other suggestions which must be dealt with before it can be con-
at there are vascular fibres to the tongue in the CT One is that the vasodilatation
rmal rat, though it was recorded in the lingual artery did not occur in the tongue
me other structure. The main candidates seem to be glandular structures. However
salivary glands, the submandibular and sublingual glands, could not be the cause,
they are not supplied by the lingual artery (Greene 1968, own observations). There
nds in the posterior part of the tongue, von Ebners glands, but they are small and
rther the hyperemia was observed in the anterior part of the tongue. It might then
ested that asoactive substances were released from them and then carried to the
r part of the tongue. This also seems highly improbable from both anatomical and
ogical points of view because it would necessitate a quite different anatomy of the
r system of the tongue from the one described (*cf* Hellekant 1972, 1976). If this was
e, then the veins from these tiny glands should drain into the arteries of the lingual
e. This is not the case. Therefore, it seems unlikely that the increased blood flow in
se to stimulation of the normal CT was caused by increased blood flow through these
ses or an effect of activity in these structures.

lly it can be suggested that the vessels in the tongue of the rats whose facial nerve had
ut already were maximally dilated before their CT was stimulated. This might then
n the absence of an effect. However this explanation seems improbable for two reasons.

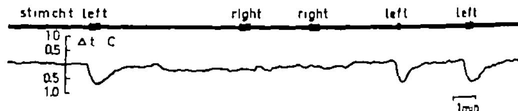


Fig. 4. The difference in temperature between the right and left side of the tongue has been recorded i rat whose right facial root had been cut. The temperature was recorded in such a ay that an upw deflection of the lower trace indicates an increase in temperature of the right side and a downward one increase on the left side. The top trace shows from the left one stimulation of the left CT two stimulati of the right CT and two stimulations of the left CT 40 Hz 10 μA. The record shows that no change of tongue temperature could be elicited on the right side of the tongue.

Further the application of this method allowed us to compare the effect of cutting facial root and having it intact in the same animal. Fig. 4 shows an example of this. In t rat the right facial root had been cut while the left side was normal. A week after the cutti the chorda tympani nerves on both sides were dissected. Their neural response to t stimulation was recorded. It looked normal Then the thermocouple was attached to tongue and connected to the recorder in such a way that a downward deflection of the tr in Fig. 4 illustrates an increase of temperature on the left side, while an upward deflect shows an increase of temperature on the right side. The signal above indicates stimulatio the chorda tympani nerves 40 Hz, 10 μA and 2 ms duration. The record in Fig. 4 shows t stimulation of the left chorda tympani nerve increased the temperature of the same side the tongue, while stimulation of the right nerve—the side on which the facial root had b cut—caused no change of surface temperature. These experiments have been repeate 5 animals with consistent results.

In conclusion, the results that have been described show that electrical stimulation of peripheral part of the CT lowered the vascular resistance of the tongue, thereby confirm earlier observations by Hellekant (1970, 1972). Further the results indicate a hyperemi the stimulated side of the tongue during the stimulation Finally recordings from the surf of the tongue showed an increase in temperature on the stimulated side of the tongue. Th effects disappeared after the facial nerve had been cut between the CNS and the genicu ganglion a week to several months before the stimulation

Discussion

It should perhaps be pointed out that this study is intended to be the first in a series wh will deal with the vascular innervation of the tongue. The main purpose of this study to rule out the explanation that the vasodilatation, which can be observed during stimulat of the CT was the result of antidromic impulses in its sensory fibres, and to show that it caused by nerve fibres to the vessels in the tongue. But before this conclusion can be dra there are a number of factors which must be considered. The first is the method used separate the sensory fibres from the motor fibres in the CT

The CT is a branch of the facial nerve. It contains sensory fib ich mediate taste

ANT, G., The influence of the circulation on taste receptors as shown by the summated chorda tympani nerve response in the rat. *Taste and Smell in Vertebrates* 1970. 83–97
ANT, G. The effect of changes in the blood flow to the tongue on the chorda tympani response in rat. *Acta physiol. scand.* 1971, 82 145–153.
ANT, G. On the relation between the chorda tympani nerve response, arterial oxygen tension and blood flow in the tongue of the rat. *Acta physiol. scand.* 1971 82. 453–459
ANT G. Circulation of the tongue. *Wenner-Gren Center International Symposium on Oral Physiology* 1972. 127–137
ANT, G. Influences on the impulse pattern in efferent chorda tympani nerve fibres in the rat. *Olfaction and Taste IV* 1972. 308–315.
ANT G. The blood circulation of the tongue. *Front. Oral. Physiol.* S. Karger 1976. 2, 130–145
ANT, G. and V GOPAL, Depression of taste responses by local or intravascular administration of ... in the rat. *Acta physiol. scand.* 1975, 95 284–292.
..., E *Die periphere Innervation.* Wilhelm Engelmann Verlag, 1964. 12th ed. 219 p.
..., A *Sur la reproduction des nerfs et sur la structure et les fonctions des ganglions spinaux. Müllers Arch. Physiol. Wiss. Med.* 1852. 392–401.

Firstly the perfusion flow through the tongues of these rats had not to be significa higher than in the normal rats to maintain the blood pressure in the lingual artery Second when a lethal dose of barbiturate was injected there was a large drop in the vascular sistance of the tongue in these rats. This indicates that these vessels were not dilated maximum as the result of the denervation

It seems from the above that there are specific nerve fibres to the vessels of the ton which when stimulated dilate vessels. The only difficulty with regard to this conclus seems to be the fact that recordings in some of these animals showed a decreased vasc resistance in the tongue to CT stimulation after the facial root was cut, though the decre was small and only observed at the highest stimulus frequencies. The explanation for th unknown There are several possibilities. Since we observed that stimulation of the ling proper nerve could have similar effects, this may indicate that antidromic impulses in sens fibres may be able to elicit a slight effect provided the stimulation is strong enough. In animal in which the maximum effect was observed (10%) some efferent nerve activity co be recorded from the central part of its CT which indicates that some regeneration mi have occurred. Another explanation is that after section all efferent fibres had not comple degenerated in these rats. Finally we (Farbman and Hellekant, to be published) have co pared the number of fibres in the part of the CT which runs along the tympanic membr and the part immediately before the nerve joins the lingual proper nerve. Though on average there was no significant difference, data from some animals undoubtedly show their peripheral CT receive a contribution of fibres whose origin has to be determined.

Any of the possibilities discussed above may be the cause for this slight vascular e which could be elicited after facial denervation in some of the rats. However it is felt this does not make the conclusion that there are specific vasodilator fibres to the vessel the tongue in the CT nerve invalid.

References

BERNARD C., De l'influence de deux ordres de nerfs qui déterminent les variations de couleur du veineux dans les organes glandulaires. *C R Acad Sci.* (Paris) 1858. *47* 245–253

BOUDREAU J C., B. E. BRADLEY P R. BIERER, S. KRUGER nd C. TSUCHITANI, Single units recor from the geniculate ganglion of the facial nerve of the cat. *Exp Brain. Res* 1971 *11* 461–488.

BUSKIRK, C. van, The seventh nerve complex. *J Comp. Neurol.* 1945 *82* 303–326.

ECCLES, R. and D I WALLIS, Vasomotor responses of the tongue and nose of the cat recorded by ple mography *J Physiol* (London) 1974 *241* 77 78P

ERICI, I and B. UVNÄS, Efferent nd antidromic vasodilator impulses t the tongue in the chorda-lin nerve of the cat. *Acta physiol scand.* 1952. *25* 10–14

FARBMAN, A I and G HELLEKANT Quant tated analysis of the f bre population in rat chorda tym proper nerves. Manuscript.

FOLEY J O., The sensory and motor axons of the chorda tympani *Proc Soc exp Biol.* (N.Y) 1945 262 267

FOLEY J O and F S. DUBOIS, An experimental study of the facial nerve. *J Comp Neurol.* 1943 *79* 101

FRIZELL, M Studies on the transport of axonal proteins in normal nd regenerating hypoglossal and nerves of the rabbit. Dissert. 1974 48P

GAUTVIK, K., The interaction of two different vasodilator mechanisms in the chorda-tympani actn submand bular salivary gland. *A ta ph siol scand.* 1970 *79* 188–203

GREENE, E. C., The anatomy of the rat. Hafner publishing Comp 1968. 370 p

…chematic drawing of the …and set-up.

…of the mean arterial pressure by 33 mmHg, decreased the reflex heart rate response …se in blood pressure, suggesting a reduced sensitivity or a changed set-point of the …l baroreceptor reflex. The studies cited above may indicate that impulses from intra…ic receptors, possibly in the low pressure area, modify the function of the carotid …ystem. In every day life man is subjected to changes in intrathoracic blood volume. …it was considered of interest to study whether changes in intrathoracic blood volume …ce the effect of a standardized carotid sinus stimulus on heart rate and blood pressure …ting capacity

…ional. Eighteen healthy male volunteers participated in the study. Mean values ±S.D. for age, … and height were 30.5±6.4 years, 77.9±8.9 kg and 1.80±0.10 m.

…cedure and methods. Details concerning catheterization procedure, measurements of intraarterial …re (P_{Bra}), central venous pressure (CVP) and heart rate (HR) are given in another article (Bevegård, …ders and Lindblad 1977). In diagrams systolic, diastolic and mean arterial pressures are abbre…d to SAP, DAP and MAP respectively.

…otid sinus stimulation. The carotid arterial stretch receptors were stimulated by applying sub…spheric pressure in a plexiglass box enclosing the neck airtightly (for technical details see Bevegård …Shepherd 1966). The dome pressure was varied by means of a servosystem operated via a function …ator. In this way it was possible to change the pressure in the box in a sinusoidal manner between … and 40 mmHg with a fixed frequency of 0.03 Hz to achieve a standardized carotid stimulus … This frequency was chosen as it has been shown to induce maximal changes in heart rate and …al pressure (Bevegård *et al.* to be published).

…hanges in blood volume distribution. The lower body (below the iliac crest) was enclosed in an air-tight … In 9 subjects the pressure was changed to 40 and 40 mmHg respectively in relation to ambient …ssure. The pressure was then kept constant during the stimulation period. These procedures will be …rred to as lower body negative pressure (LBNP 40) and lower body positive pressure (LBPP +40). …10 subjects the box pressure was reduced in steps of 20 mmHg every third minute. Details concerning …NP and LBPP are given in a separate article (Bevegård *et al.* 1977).

…ignal analysis. The tape-recorded physiological output signals, i.e. ECG and blood pressure, were …lyzed beat to beat off-line on a digital computer. The periodic variations in heart rate and systolic, …stolic and mean arterial pressure induced by variations in the effective transmural pressure (mean …terial pressure minus dome pressure) were then examined. Briefly the computer calculated the amplitude

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…These subjects also participated in hemodynamic studies, which are presented in separate articles …evegård *et al.* 1977; Bevegård, Castenfors and Lindblad to be published).

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Blood Pressure and Heart Rate Regulating Capacity of the Carotid Sinus during Changes in Blood Volume Distribution in Man

By

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Abstract

BEVEGÅRD S., J CASTENFORS L. E. LINDBLAD and J TRANESJÖ. *Blood pressure and heart rate regulating capacity of the carotid sinus during changes in blood volume distribution in man* Acta physiol. scand. 1977 99 300-312.

The influence of changes in blood volume distribution on the carotid baroreflex was studied in subjects. Blood volume distribution was changed by varying the pressure around the lower body above and below ambient, thereby varying the amount of blood pooled in this region and exerting a second influence on the central blood volume. The carotid arterial stretch receptors were stimulated by varying the pressure in an air-tight box enclosing the neck. To obtain a standardized carotid sinus stimulus (SCS) the pressure in the box was varied sinusoidally between −10 and 40 mmHg with a frequency of 0.03 Hz. The effect on heart rate and blood pressure were assessed by harmonic analysis performed off-line on a digital computer. During lower body negative pressure of 40 mm (LBNP −40), *i e* during a procedure known to reduce the central blood volume, SCS induced an augmented effect on the blood pressure regulating capacity but not on the heart rate response. Expressing the blood pressure regulating capacity as peak-to-peak changes in systolic arterial pressure, the response during LBNP −40 mmHg was almost twice the control value. The opposite stimulus—lower body positive pressure—influenced the SCS-induced effects only slightly but on the average a minor reduction in the blood pressure and heart rate regulating capacity was found compared with the control condition, though the difference did not reach significant levels.

The results support the hypothesis that changes in blood volume distribution modify the function of the carotid baroreflex, possibly via intrathoracic receptors sensitive to changes in central blood volume and/or central venous pressure.

Key words: Blood volume, carotid sinus, blood pressure, heart rate

Experiments in rats by Castenfors and Sjöstrand (1973) have demonstrated that hypervolemia decreases the blood pressure response to bilateral carotid occlusion while a moderate hypovolemia tends to increase the blood pressure response to this stimulus. Koike *et al* (1975) demonstrated in anesthetized dogs an augmented gain of the carotid baroreceptor reflex after interruption of cardiopulmonary afferents. In a recent study Vatner *et al* (1975) working with unanesthetized dogs, demonstrated that an acute volume load resulting in a

ble 1. Effect of LBNP −40 (n = 6) and LBPP +40 (= 8) on intravascular pressures and heart rate. Mean values ± S.E.

	Control	LBNP 40	Diff.	Sign. of diff.	Control	LBPP +40	Diff	Sign. of diff.
art rate	71.8	83.7	+11.8	$p<0.01$	69.3	70.3	+1.0	s.
beats·min⁻¹)	±1.7	±3.1	±1.8		±2.6	±[illegible]	±1.6	
stolic arterial pressure	129.7	127.3	−2.3	s.	132.8	136.8	+4.0	s.
mmHg)	±3.2	±3.7	±1.9		±3.4	±4.6	±2.7	
ean arterial pressure	93.5	93.8	+0.3	s.	94.6	99.0	+4.4	$p<0.05$
mmHg)	±2.5	±2.9	±0.1		±2.3	±2.6	±1.2	
astolic arterial pressure	80.2	85.0	+4.8	$p<0.05$	82.6	85.6	+3.0	s.
mmHg)	±1.6	±1.6	±1.7		±1.3	±2.5	±1.4	
entral venous pressure	6.5	2.1	−4.3	$p<0.01$	7.5	8.8	+1.3	n.s.
mmHg)	±1.0	±1.3	±0.5		±0.7	±1.0	±0.6	

I two subjects LBNP 40 caused a progressive fall in blood pressure, resulting in an mpending syncope which necessitated an abrupt termination of the stimulus. These two subjects have been excluded from the analysis. In the 6 subjects who maintained a relative irculatory steady state, LBNP induced a significant increase in heart rate and diastolic rterial pressure, while systolic arterial pressure and mean arterial pressure were not significantly changed. Central venous pressure decreased significantly (Table 1).

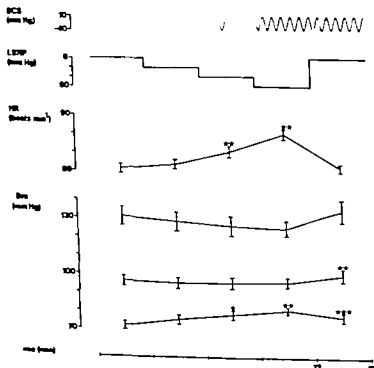


Fig. 1. Effect of gradually increasing LBNP stimulation on heart rate and blood pressure (mean values SEM). The asterisks give the level of significance for difference compared with control.

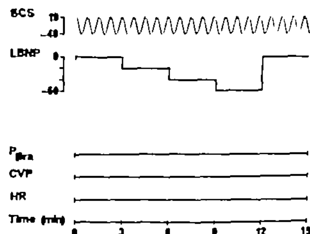


Fig. 2a.

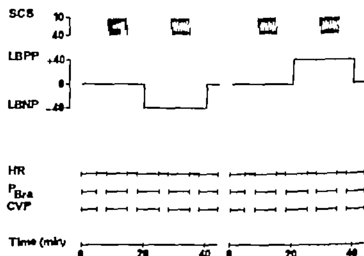


Fig. 2b.

Fig. 2 a and b. Schematic presentation of applied stimuli and recorded variables.

of the sine wave that best f tted the resulting changes in heart rate and P_{Bra} thus yielding information about the dynamic features of the baroreceptor system. Results concerning the central ... were calculated manually Details of the analyses and calculation of the baroreceptor system gain are given in the Appendix

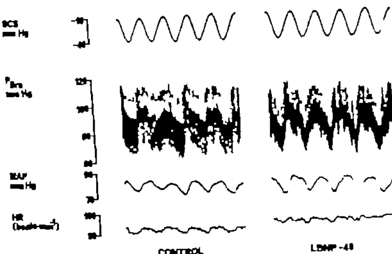
Statistical methods The differences between dat obtained during different blood volume distributions in each subject were analysed with Student's t test f paired data. Results are designated as follows: $p<0.05$ x, $p<0.01$ xx, $p<0.001$ xxx. P values <0.05 re considered significant.

General experimental design. A schematic presentation of the experimental set-up is given in Fig. The measurements were made with the subjects lying supine in room with a temperature of 22°C. After the introduction of the catheters, the boxes surrounding the neck nd the lower body mounted. The subjects were then allowed to rest for bout 10 mi before starting the measurements. Two experimental designs were used as described n Fig 2 a and 2 b respecti ely The data on the dynamic effects of LBNP and LBPP per se refer to mean values of measurements before and after as indicated i Fig. 2 b.

Results

Effects of LBNP −40 and LBPP +40 on intravascular pressures and heart rate

The circulatory effects of LBNP −40 and LBPP +40 respectively in these subjects presented and discussed in detail in a separate article (Bevegård *et al* 1977).



5 Segment of an original recording during control condition and LBNP −40.

5 In none of the measured dynamic variables (i.e. peak-to-peak variation of heart , systolic arterial pressure, mean arterial pressure and diastolic arterial pressure) could gnificant difference be demonstrated between the recordings during the two control iods in the same individual.

During LBNP −40 During LBNP −40 there were increases in peak-to-peak changes in tolic arterial pressure, mean arterial pressure and diastolic arterial pressure, though the rease reached significant levels only in the first two variables (Table II). In most cases heart rate response was attenuated during LBNP −40, when several subjects displayed nost no sine wave fluctuation in heart rate (Fig 5).

During LBPP +40. Comparing the findings during LBPP +40 with those during the preding control period showed slight but not significant decrease in the baroreceptor ediated peak-to-peak changes in blood pressure as well as heart rate (Table II).

During gradually increasing LBNP stimulation. Increasing the amount of blood pooled the lower body by gradually decreasing the pressure surrounding this region, resulted in

ABLE II Amplitude (peak-to-peak changes) in heart rate and blood pressure induced by SCS under control conditions and during LBNP −40 (n = 6) and LBPP +40 (n = 8) Mean values ± S.E.

	Control	LBNP −40	Diff.	Sign. of diff	Control	LBPP +40	Diff	Sign. of diff.
Heart rate	9.1	7.0	2.2	n.s	9.4	8.7	−0.7	n.s.
(beats min⁻¹)	±1.7	±2.5	±1.7		±2.2	±3.1	±1.2	
Systolic arterial pressure	6.0	11.0	5.0	$p<0.01$	8.8	7.6	1.2	
(mmHg)	±1.0	±1.2	±1.0		±2.3	±1.2	±1.3	
Mean arterial pressure	6.8	10.4	3.6	p 0.05	8.5	8.0	−0.5	n.s.
(mmHg)	±1.1	±1.4	±1.0		±1.8	±1.3	±0.9	
Diastolic arterial pressure	6.8	8.7	1.9	n.s.	7.5	7.2	−0.3	
(mmHg)	±0.9	±1.8	±1.3		±1.6	±1.2	±1.1	

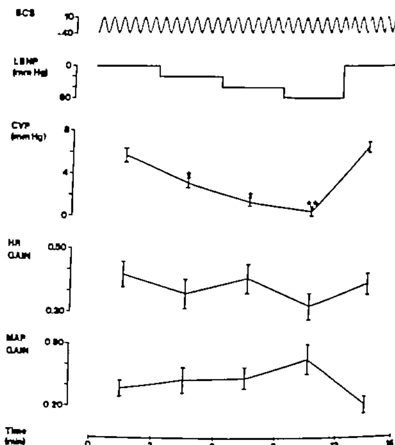


Fig. 4 Effect of gradually increasing LBNP stimulation on central venous pressure, heart rate (HR gain) and mean arterial pressure gain (MAP gain). Mean values ±SEM. The asterisks denote the ... of significance for difference compared with control. (Details of the calculation of the gain factors are ... in Appendix.)

LBPP +40 resulted in a slight but significant increase in mean arterial pressure, whe... the slight increases in systolic and diastolic arterial pressure were not statistically signific... Heart rate was essentially unchanged. (Table I.) A significant increase in central ve... pressure (+2.1 mmHg, $p < 0.05$) was found initially but central venous pressure gradu... decreased towards the control value and during the period of SCS only a slight mean ... crease persisted, which was not statistically significant.

Circulatory effects of gradually increasing LBNP stimulation

Only data obtained during a simultaneous continuous SCS are presented. The hemodyna... changes induced were basically similar to those during LBNP −40. The magnitude of ... changes increased gradually with increasing LBNP stimulation. (Fig. 3, Fig. 4.)

The sudden release of LBNP elicited a transient "overshoot" in central venous press... amounting to 1.4 mmHg ($p < 0.05$) above the control value.

Carotid sinus stimulation—effects on blood pressure and heart rate

During control conditions. SCS in all cases caused blood pressure and heart rate to vary approximately sinusoidally with the same frequency as the input signal, as illustrated in

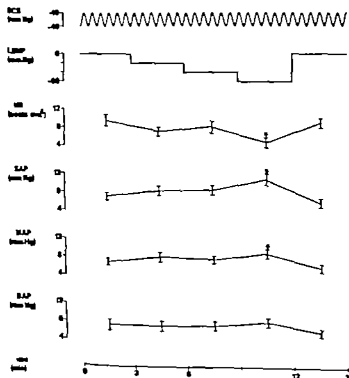


Fig. 7. Peak-to-peak changes in heart rate and blood pressure (Mean values ±SEM) during gradually increasing LBNP stimulation. The asterisks give the level of significance for difference compared with control.

by a linear relation between dome and esophageal pressure. Kober *et al.* (1970) using an ultrasonic technique, demonstrated—for the pressure used in the present study—a linear relation between the increase in transmural pressure and the resulting increase in the radius of the carotid artery.

By using sinusoidally varying dome pressure, the effect could be calculated as the mean of several identical stimulations, thereby increasing the precision of the measurements. This stimulation method also has the obvious advantage of being suitable for harmonic analysis, using a computer as described in Appendix. As a consequence of the technique, however the results of the analysis must be interpreted with caution the more the output signals deviate from the ideal sine wave (see Appendix).

Changing the pressure around the lower body is a well-documented method for changing the amount of blood pooled in the lower body thus influencing the central blood volume. The hemodynamic effects of LBNP and LBPP in these subjects are discussed in a separate article (Bevegård *et al.* 1977).

The effects demonstrated on heart rate and blood pressure by SCS during control conditions in this study correspond well to earlier reports using neck suction as baroreceptor stimulation (Bevegård *et al.* 1966, Thron *et al.* 1967, Stegemann *et al.* 1970, Bjurstedt *et al.* 1975 and Abboud *et al.* 1975).

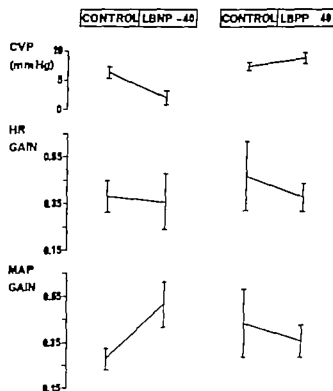


Fig. 6. Central venous pressure, gain (HR gain) and mean arte pressure gai (MAP gain) duri condition, LBNP −40 and LI (Mean values ±S.E.). Asteris significance of deviation. (Det calculation of the gain factors in Appendix.)

a progressive decrease of central venous pressure and on the average, a slight pr increase in peak-to-peak changes of the blood pressure (Fig. 7), whereas the heart sponse became attenuated and distorted (Fig. 7 Appendix Fig. 8)

Baroreceptor system regulating capacity If changes in baroreceptor blood pressure ing capacity are interpreted in terms of changes in the system gain factor (Appendix average a slight though not significant decrease was found during LBPP (Fig. 6), in to a significant increase during LBNP −40 During gradually increasing LBNP stim a significant increase was found during LBNP −40 and LBNP −60 (Fig. 4).

In the period shortly after the release of the LBNP stimulus, however the blood regulating capacity expressed as the mean arterial pressure gain factor showed a si decrease compared with the control condition (Fig. 4)

If changes in heart rate regulating capacity induced by the carotid barorecep expressed in terms of system gain factors, no significant changes were found duri LBNP −40 or LBPP +40 though a significant decrease was found during LBNP

Discussion Stimulation of the carotid arterial stretch receptors in man by apply atmospheric pressure around the neck was first used by Ernsting and Parry in similar technique has been used since then by several authors (Bevegård and Shephe Thron *et al* 1967 Wagner Wacherbauer and Hilger 1968 Beiser *et al*. 1970, Ko Arndt 1970 Stegemann Busert and Brock 1974 Abboud *et al* 1975 Bjurstedt, hamer and Tydén 1975)

The technique has been evaluated by for instance Thron *et al* 1967 who showed pressure applied was transmitted fairly well to the upper part of the esophagus, as e

ggest that the blood pressure regulating capacity is increased during LBNP as evidenced y the significant increase in mean arterial pressure gain factor

During LBNP the heart rate response became distorted, which means that the results concerning the heart rate regulating capacity during LBNP have to be interpreted with care (see Appendix). However it may at last be concluded that there is no indication of an increase in the baroreceptor mediated heart rate response.

During LBPP on the average, the blood pressure and heart rate response showed a slight, non-significant decrease. However LBPP induced only a transient increase in central venous pressure, and during the SCS period no significant increase in central venous pressure was found compared with control conditions. This may indicate that the increase in central blood volume was only minor during the SCS period and thus contributed to the finding of a small non-significant decrease in blood pressure and heart rate response to SCS. This is supported by the observation that after the sudden release of LBNP stimulus with simultaneous SCS, there was a significant decrease in the mean arterial pressure gain factor compared with control conditions. On release of the LBNP stimulus one would expect a rapid increase in central blood volume, possibly influencing receptors in the low pressure area (Brown *et al.* 1966), i a hemodynamic situation similar to that caused by the onset of LBPP

With the present data it is not possible to assess a conceivable modifying effect of the aortic baroreceptors on the effects of SCS under different experimental conditions. Data on the aortic baroreflex in man are very limited in the literature. However in anaesthetized dogs the aortic baroreflex has been shown to respond mainly to changes in mean arterial pressure and only weakly to changes in pulse pressure (James and de Burgh Daly 1970) It has also been suggested that the aortic baroreflex is of importance mainly in a pressure range above the normal level (Donald and Edis 1971 Pelletier Clement and Shepherd 1972). If these results can be extrapolated to man, it is unlikely that under the conditions in this study in which mean arterial pressure deviated only slightly from control values, the effects of stimulating the carotid sinus would be influenced at all substantially by changes in the aortic baroreceptor activity

A modifying effect of changes in arterial pressure, dp/dt, pulse pressure or heart rate on the carotid sinus cannot be excluded. If the findings in dogs are attributable to man, intrasinus pressure (*i.e.* mean intraarterial pressure) is the single most important determinant, whereas, in the physiological range, variations in pulse pressure and, particularly heart rate have relatively little reflex effect (Schmidt, Kumada and Sagawa 1972). In the present study during SCS the mean blood pressure during LBNP 60 deviates from the control level by only 1 mmHg. It is therefore unlikely that changes in mean arterial pressure account for the significant increase that was demonstrated in baroreceptor sensitivity during LBNP

Evidence that pooling of blood in the lower body decreases the filling pressure of the heart, i the transmural pressure, has been presented by Murray *et al.* (1968). They demonstrated that even LBNP 60 caused only minor and inconsistent decreases in intrathoracic pressure as assessed from the esophageal pressure (range 0–2.2 mmHg), whereas central venous pressure showed a consistent decrease of the order of 7 mmHg.

This suggests that LBNP ought to provide an adequate stimulus for vascular receptors

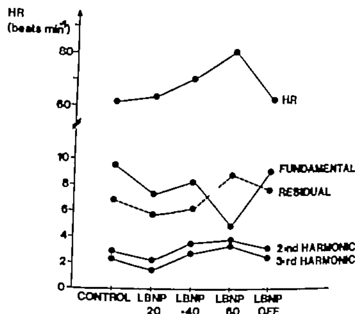


Fig. 8a

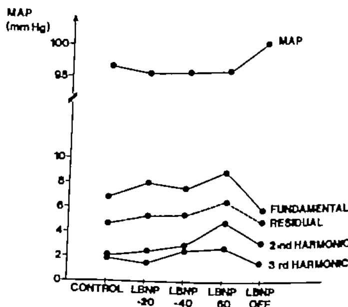


Fig. 8b

Fig. 8. Amplitudes (peak-to-peak values) of fundamental second and third harmonic sinusoidal comp nents during increasing LBNP stimulation. A Heart rate. B Mea arterial pressure. To make t relation between the fundamental and residual, the latter is expressed in effective value. As a referen mean values of mean arterial pressure and heart rate are also given (top of diagrams).

The hemodynamic changes induced by LBNP −40 resulted in a significant increase i the baroreceptor mediated blood pressure response and during gradually increasing LBN stimulation there was a progressive increase in the blood pressure response. Although th carotid sinus blood pressure buffering system is not an ideally linear function, the result

...pal quality

The fluctuations of heart rate and systolic, mean and diastolic pressures in response to SCS were, as expected, basically sine-wave shaped. However, harmonics and "noise" were included. In Fig. 8 the relationships between the fundamental sine wave, the first two harmonics and the residual are shown for heart rate and mean arterial pressure during increasing levels of LBNP. Results are based on the closed-loop system. Results of the same magnitude range were obtained for LBNP −40 and LBPP +40.

As demonstrated, the residuals and the fundamentals are of the same magnitude. The relatively large residuals are interpreted as partly a consequence of the normal physiological fluctuations in heart rate and MAP. For example, the normal sinus arrhythmia in resting young men has a magnitude of approx. 6–7 beats/min (Schlomka 1937) and the same frequency as respiration, i.e. 10–15 periods/minute. This frequency corresponds to the 5th–8th harmonics in this study, thus augmenting the residual. The second and third harmonics are only 5 times smaller than the fundamental, indicating the non-linearity of the baroreceptor system.

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References

Abboud, F. M., A. L. Mark, D. D. Heistad, D. L. Eckberg and P. G. Schmid, Selectivity of autonomic control of the peripheral circulation in man. *Trans. Amer. clin. climat. Ass.* 1975. 86. 184–197.

Beiser, D. G., R. Zelis, S. E. Epstein, D. T. Mason and E. Braunwald, The role of skin and muscle resistance vessels in reflexes mediated by the baroreceptor system. *J. clin. Invest.* 1970. 49. 225–231.

Bevegård, S. B. and J. T. Shepherd, Circulatory effects of stimulating the carotid arterial stretch receptors in man at rest and during exercise. *J. clin. Invest.* 1966. 45. 132–142.

Bevegård, S. B., J. Castenfors and L. E. Lindblad, Effect of carotid sinus stimulation on cardiac output and peripheral vascular resistance during changes in blood volume distribution in man. Submitted for publication to *Acta physiol. scand.*

Bevegård, S. B., J. Castenfors and L. E. Lindblad, Effect of changes in blood volume distribution on circulatory variables and plasma renin activity in man. *Acta physiol. scand.* 1977. 99. 237–245.

Bevegård, S. B., J. Castenfors, M. Danielsson, L. E. Lindblad, G. Rosenhamer, J. Tranmöd and O. Wigertz, Analysis of the dynamic function of the carotid sinus reflex in man. To be published.

Bjurstedt, H., G. Rosenhamer and G. Tydén, Cardiovascular responses to changes in carotid sinus transmural pressure in man. *Acta physiol. scand.* 1975. 94. 497–505.

Bromander, S. and O. Wigertz, Beskrivning av fortranprogram för datamaskinell utvärdering av fysiologiska mätvärden. Int. Report. Department of Aviation and Naval Medicine, the Karolinska Institute, Stockholm, Sweden. 1967.

Brown, E., J. S. Goei, A. D. M. Greenfield and G. C. Plassaras, Circulatory responses to simulated gravitational shifts of blood in man induced by exposure of the body below the iliac crests to sub-atmospheric pressure. *J. Physiol.* (Lond.) 1966. *183.* 607–627.

Castenfors, J. and T. Sjöstrand, Circulatory control via vagal afferents. IV. Integration of the central control mechanisms in the circulatory adaptation to variations of blood volume. *Acta physiol. scand.* 1973. 87. 188–198.

Donald, D. E. and A. J. Edis, Comparison of aortic and carotid baroreflexes in the dog. *J. Physiol.* (Lond.) 1971. *215.* 521–538.

Elgerd, O. *Control systems theory.* McGraw-Hill, New York. 1967.

Ernsting, J. and D. J. Parry, Some observations of the effects of stimulating the stretch receptors in the carotid artery of man. *J. Physiol.* (Lond.) 1957. *137.* 45 p–46 p.

James, J. E. A. and M. de Burgh Daly, Comparison of the reflex vasomotor responses to separate and combined stimulation of the carotid sinus and aortic arch baroreceptors by pulsatile and non-pulsatile pressures in the dog. *J. Physiol.* (Lond.) 1970. 209. 257–293.

K[illegible], G. and J. O. Arndt, Die Druck-Durchmesser Beziehung der A. Carotis Communis des Wachen Menschen. *Pflügers Arch. ges. Physiol.* 1970. *314.* 27–39.

Koike, H., A. L. Mark, D. D. Heistad and P. G. Schmid, Influence of cardiopulmonary vagal afferent activity on carotid chemoreceptor and baroreceptor reflexes in the dog. *Circulat. Res.* 1975. 37. 422–429.

Murray, R. H., L. J. Thompson, J. A. Bowers and C. D. Albright, Hemodynamic effects of graded hypovolemia and vasodepressor syncope induced by lower body negative pressure. *Amer. Heart J.* 1968. 76. 799–811.

on the venous side that are sensitive to changes in transmural pressure. Thus the findings i this study might be interpreted as supporting studies mentioned earlier by Castenfors *et a* (1973) Vatner *et al* (1975) and Koike *et al* (1975), suggesting that impulses from intrathorac receptors, possibly in the low pressure area, modify the function of the carotid sinus syste

Appendix

Signal analysis

(The electrocardiogram (ECG), the brachial artery pressure (P_{Bra}) and the dome pressure were reco on an analog FM tape recorder (Tandberg, model 100) for subsequent off-line computer (Nord analysis. Heart rate (HR) as well as systolic, diastolic and mean pressures were calculated beat-to-b (Nygårds *et al.* 1976). To obtain steady state values, computer analysis was always started at least 1 after a change in external conditions.

Time equidistant signal values are required as input of the final harmonic analysis described bel Therefore, HR and P_{Bra} were computed at 1 s intervals assuming linear changes between consecu beat-to-beat values.

According to control theory harmonic analysis (Elgerd 1967) of a control system can be used to de system features if the input and output signals are known. With a sine wave (amplitude A, frequency

$$x(t) = A \sin 2\pi ft + C_1$$

as input, the output y(t) will generally be a sum of sine waves

$$y(t) = \sum_{1}^{\infty} B_n \sin (n\ 2\pi ft + \phi_n) + C_2$$

where B and ϕ represent the amplitude and phase lag of the *n*th *harmonic* wave with the frequ n f C_1 and C_2 are the offset (DC) values of the input and output signals respectively Note that the fu mental output wave (n = 1) has the same frequency as the input.

For a linear system, all harmonics equal zero. If the fundamental is large compared with the harmo the non-linear system could be approximated as linear (within certain limits)

A computer program for harmonic analysis (Broman and Wigertz 1967) was used in this study computer calculated the sine wave that best described the recorded fluctuations in the output sig *i.e.* heart rate and blood pressures. In addition, the first two output harmonics were calculated information loss by using the fundamental and its first two harmonics to describe the results is given "residual" which is presented here as the effective value of remaining harmonics and spontan fluctuations ("noise")

A total of four sine wave periods were A/D-converted. Due to the relatively high "noise" level of output signal (Appendix Fig. 8) the three initial and the three final periods were analysed independe The results presented here are based on the three periods having the smallest residual, thus giving best description of the output signal.

Gain factor

To verify that the demonstrated effects depend on a real change i buffering capacity control theory ca applied by regarding the reflex as a closed loop system which is disturbed by the pressure surroundin sinus wall (Stegemann *et al.* 1974).

Assuming that intrasinus blood pressure equals the pressure measured i the brachial artery and tha subatmospheric pressure applied around the neck is almost completely transmitted to the sinus all (quoted in a review by Wolthuis, Bergman and Nicogossian 1974 and Kober *et al.* (1970)) the effe transmural pressure (TMP) was calculated as the difference between mean arterial pressure and d pressure.

In the computerized harmonic analysis, the gain factor (defined as the amplitude of the output s harmonic sine wave divided by the amplitude of the input sine wave, *i.e.* B_1/A according to eq 1 w of heart rate and MAP was first calculated regarding the dome pressure as input signal (closed loop g By transforming the results with regard to control theory (Elgerd 1967), the gain factors were obta regarding the TMP as system input (open loop gain).

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Changes in ATP and Cyclic Nucleotide Levels during Sympathetic Nerve Stimulation in Canine Subcutaneous Adipose Tissue in situ[1]

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Abstract

FREDHOLM, B. B., E. BELFRAGE and E. BLASCHKE. *Changes in ATP and cyclic nucleotide levels in canine subcutaneous adipose tissue during sympathetic nerve stimulation.* Acta physiol. scand. 1977. 99. 313–322.

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SCHLOMKA, G Untersuchungen über die physiologische Unregelmässigkeit des Herzschlages *Z. Kreisl Forsch.* 1937 *29* 510–524

SCHMIDT R M M KUMADA and K. SAGAWA, Cardiovascular responses to various pulsatile pressures in the carotid sinus. *Amer J Physiol.* 1972, *223* 1 7

STEGEMANN, J A. BUSERT and D BROCK, Influence of fitness on the blood pressure control system in man. *Aerospace Med* 1974 *45* 45–48

TIRON, H L., W BRECHMAN, J WAGNER and K. KELLER, Quantitative Untersuchungen über die Bedeutung der Gefässdehnungsreceptoren im Rahmen der Kreislaufhomoiostase beim Menschen. *Pflügers Arch ges. Physiol* 1967 *293* 68–99

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WAGNER, J J WACHERBAUER and H H. HILGER, Arterielles Blutdruck und Herzfrequenzverhalten bei Hypertonikern unter Änderung des transmuralen Druckes im Karotissinusbereich. *Z. Kreisl-Forsch* 1968 *57* 701–712.

WOLTHUIS, R. A., S. A. BERGMAN and A. E. NICOGOSSIAN, Physiological effects of locally applied reduced pressure in man. *Physiol. Rev* 1974 *54* 566–595

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From the Department of Pharmacology Karolinska Institutet, Stockholm, Sweden

Changes in ATP and Cyclic Nucleotide Levels during Sympathetic Nerve Stimulation in Canine Subcutaneous Adipose Tissue *in situ*

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SCHMIDT R. M M. KUMADA and K. SAGAWA, Cardiovascular responses to various [illegible] in the carotid sinus. *Amer J Physiol.* 1972. *223* 1–7

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Cyclic AMP was measured on 50 μl aliquots of plasma or neutralized tissue extracts by competitive binding assay (Brown *et al.* 1972). The binding protein was prepared from bovine adrenal cortex. ^{3}H-cyclic adenosine 3',5'-monophosphate (27.5 Ci/mMol) was obtained from the Radiochemical Centre, Amersham, England and unlabelled cyclic AMP from Boehringer Mannheim, West-Germany. Recovery of ^{3}H-cyclic AMP added at the homogenization step was essentially complete. Purification of the samples was not necessary (Fernebo *et al.* 1976).

Cyclic GMP was measured by radioimmunoassay according to Steiner *et al.* (1972) as modified by Harper and Brooker (1975). 2'-O-succinyl-cyclic guanosine 3',5'-monophosphate (Sigma, St. Louis, USA) was coupled to keyhole limpet hemocyanin (Calbiochem, USA) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The antigen was injected subcutaneously into rabbits every 3–6 weeks to generate the antibodies. The antibodies were used at final dilution of 1 20 000 to 1 50 000. Unlabelled cyclic GMP (Boehringer, Mannheim), samples or blanks were treated with acetic anhydride in triethylamine to acetylate the cyclic nucleotide. Incubation for 3 h at 4°C using ^{125}I-tyrosyl-succinyl cyclic GMP (Schwarz-Mann, Orangeburg, USA) as the labelled antigen. Free and bound cyclic GMP was separated by the addition of 4 volumes of 60 saturated ammonium sulfate. Amounts of cyclic GMP between 0.1 and 5 pMol were determined with precision of 8 per cent. Cyclic AMP AMP ADP ATP GDP GTP did not interfere with the assay at 1 000 times higher concentration. Purification of the samples on short columns Dowex 50X-8 did not alter results.

ATP was determined by the luciferine-luciferase method essentially as described by Stanley and Williams (1969). The enzymes were extracted from desiccated tails and lanterns of fireflies (Sigma) with 1 M sodium arsenate buffer pH 7.4 one day in advance to minimize blank readings. The assay was performed at room temperature in 1.9 ml final volume of 50 mM sodium arsenate, 25 mM magnesium sulfate and 0.2 ml of diluted tissue extract. The reaction was started by the addition of firefly extract. After 6 the Packard 2000 liquid scintillation spectrometer was started to count the emitted photons for a period of 6. The optimal setting was found to be: out of coincidence, 100 amplification and 40–60 window setting of the discriminator. Sample readings (in duplicate) were compared with standards (1 50) pmol. It has been reported that ATP coprecipitates with potassium perchlorate (Wiener *et al.* 1974). The loss of ATP may be appreciable at low ATP levels and at high concentrations of perchloric acid. Under our conditions the recovery of ^{3}H-ATP in the supernatant after precipitation was 85–98 per cent. Corrections for this loss were not carried out.

Statistics. Results are expressed as mean ± S.E. and hypotheses were tested by the Mann-Whitney test or the Student's *t*-test.

Results and discussion

Nucleotide levels under basal conditions

The level of ATP found in biopsies from canine subcutaneous adipose tissue, 73.6 ± 6.7 nmol/g w.w. (n 21) agrees with that reported for other species (30–110 nmol/g w.w., Denton *et al.* 1966, Hepp *et al.* 1968, Bihler and Jeanrenaud 1970, Hanson and Ballard 1970, Angel *et al.* 1971 Knight and Iliffe 1973 Fredholm and Frooek 1974). This suggests that ATP levels in adipose tissue are quite similar in different species and that the present technique has been adequate.

Similarly the value for cyclic AMP content in canine subcutaneous adipose tissue, 90.0 ± 12.0 pmol/g w.w. (n 28) found in the present study falls within the range reported for rat and human adipose tissue (37–180 pmol/g w.w. Butcher *et al.* 1965 Fassina *et al.* 1972 a, Knight and Iliffe 1973 Arner and Östman 1975). There is little reported information about the cyclic GMP content in adipose tissue. We found a cyclic GMP level of 17.7 ± 2.9 pmol/g w.w. (n 8) which is similar to the values recently reported for rat parametrial fat cells (7 18 pmol/g w.w.) by Fain and Butcher (1976). Illiano *et al.* found between 6 and 30 pmol cyclic GMP per million fat cells. The corresponding value for cyclic AMP reported by most authors is around 40 pmol/10^6 cells (*e.g.* Fredholm and Hjemdahl 1976). This corresponds

opposing effects and that the magnitude of the response may be determined by the ratio between cyclic AMP and cyclic GMP in the tissue (Goldberg *et al.* 1975). A report by Illiano *et al* (1973) on the effect of insulin and acetylcholine in isolated fat cells suggests that the lipolytic response may also be governed by the cyclic AMP/cyclic GMP concentration ratio

The relationship between ATP and lipolysis seems to be complex. ATP is necessary for the synthesis of cyclic AMP and also for the activation of hormone sensitive lipase by protein kinase (Huttunen *et al* 1970) It has been found that inhibitors of glycolysis or oxidative phosphorylation depress fat cell ATP levels and inhibit lipolysis before and after the stage of cyclic AMP formation (Hollenberger and Patten 1970, Fassina *et al* 1972 a, 1972 b). Conversely the ATP levels in adipose tissue are depressed by lipolytic hormones (Hepp *et al* 1968 Bihler and Jeanrenaud 1970, Angel, Desai and Halprin 1971). The fall in ATP under *in vitro* conditions appeared to be strongly dependent on the accumulation of intracellular fatty acids (Angel *et al.* 1971) Under *in vivo* conditions other factors may also play a role in the regulation of adipose tissue ATP levels. Thus, when vasoconstriction is induced in adipose tissue there is a pronounced fall in the tissue oxygen tension down to levels where the oxygen supply may limit ATP synthesis (Fredholm *et al* 1976).

In the present study we have examined the effect of sympathetic nerve stimulation on adipose tissue cyclic nucleotide and ATP levels under *in vivo* conditions. We report that sympathetic nerve stimulation induces a reversible fall in the ATP level and an increase in the cyclic AMP/cyclic GMP concentration ratio Some possible explanations and implications of these findings are briefly discussed.

Materials and methods

Animals. 9 fed female mongrel dogs weighi g 9–15 kg were used Anesthesia was induced by sodium pentobarb tal (Nembutal, Abbot, 25–30 mg/kg i.) and was supplemented whenever necessary Heparin 1 000–2 500 U/kg was given 30–60 min before the expt. t prevent clotti g of blood The animals were tracheotomized nd artificially ventilated.

Experimental. Subcutaneous dipose tissue was isolated (Rosell 1966) and autoperfused from femoral artery An arterial drop counter was used to monitor blood flow The venous effluent as returned to the animal via the femoral vein. Adipose tissue blood flow and systemic arterial blood pressure (Statham P23 AC) were recorded on a Grass Polygraph. Samples (1–4 ml) of arterial and venous blood were taken in t bes containing 20–50 µl 0.5 M sodium – EDTA. These tubes were kept on ice until centrifuged. Aliquots f plasma were frozen for subsequent nalysis of cyclic AMP ATP or radioactivity or were deproteinized for the assay of glycerol (see below).

The cut nerve to the adipose tissue preparation was placed on bipolar platinum (or silver) electrodes protected from drying by Plastibase (Squibb), and stimulated at the rate of 3–4 Hz with pulses of supramaximal intensity (12 15 V) and duration (2 ms) delivered by a Grass 7B stimulato

Tissue handling Biopsies f 30–150 mg of the perfused adipose tissue were rapidly cut out and frozen in liquid nitrogen. The time between the start of taking the biopsy until freezi g did not exceed 2 s. The biopsies were kept at −80°C or i liquid nitrogen until they were crushed between metal blocks precooled by liquid nitrogen. The powered material was quickly poured in tarred homogenizer tubes containing 1 ml 0.4 N PCA, weighed and homogenized. After centrifugation an aliquot of the clear layer between the precipitate and fat cak was taken and neutralized w th 4 N KOH nd sufficient 1 M Tris base to make the final concentration 0.05 M The neutralized extract was stored t −20°C until assayed.

Assays. Glycerol was measured enzymatically as described by Laurell and T bbling (1966). Results are expressed as µmol glycerol released per minute and 100 g tissue.

TABLE II Effect of sympathetic nerve stimulation on adipose tissue cyclic nucleotide levels. Mean ± S.E. Number of determination within parenthesis

	Cyclic nucleotide level in % of control			
	During stim.		After stim.	
	2–4 min[a]	7–12 min[a]	2–3 min[b]	7–12 min[b]
Cyclic AMP	114 ± 18 (9)	152 ± 31 (12) $p < 0.05$	123 ± 17 (9)	95 ± 16 (9)
Cyclic GMP	89 ± 35 (4)	44 ± 15 (6) p 0.01	146 ± 33 (7)	141 ± 37 (10)

Control cyclic AMP was 90.0 ± 12.0 (28) pmol/g w.w. and control cyclic GMP was 17.7 ± 2.9 (8) pmol/g w.w. After α-blockade cyclic AMP was 130 ± 33 (8) pmol/g, cyclic GMP 10.8 (3) pmol/g; after β-blockade values were 82 ± 13 (4) and 10.0 (2).

[a] Refers to time after the start of stimulation.
[b] Refers to time after the end of stimulation.

...ntration difference for cyclic AMP found during resting condition was enhanced, rather ... diminished or reversed, during nerve stimulation (average venous cyclic AMP 11.4 nM). ...ter stimulation the venous cyclic AMP levels returned to control. The decreased venous ... the AMP during nerve stimulation probably results from the decreased blood flow and ... consequently increased time of exposure of cyclic AMP to phosphodiesterase in plasma ... and in the formed elements. Even so, our results suggest that adipose tissue does not con...tribute significantly to the increase in plasma cyclic AMP levels seen during catecholamine ...infusion (Ball et al. 1972). In a recent study Issekutz (1975) similarly concluded that ...there is no relationship between plasma cyclic AMP turnover and FFA mobilization from ...adipose tissue. Conversely in situations of high sympathoadrenal tone, such as hemorrhage, ...there may be a large increase in plasma cyclic AMP levels with no increase in the plasma ...level of glycerol or FFA (Farnebo et al. 1976).

The results presented in Table II further show a significant fall in tissue cyclic GMP levels ...during nerve stimulation. Fain and Butcher (1976) recently reported that noradrenaline ...increases cyclic GMP levels in rat fat cells under their basal conditions. However when the ...cells were exposed to insulin, fatty acids or a calcium ionophore, which by themselves raise ...cyclic GMP, noradrenaline sometimes produced a decrease in cyclic GMP levels. Adipose ...tissue in situ is exposed to insulin and furthermore the level of FFA seems to be high (Fred...holm 1970, Kashyap et al. 1975). Therefore the difference between our results and those of Fain and Butcher may be due, at least in part, to differences in the basal conditions. Our finding of a markedly elevated cyclic AMP/cyclic GMP ratio in parallel with an enhanced lipolysis (Table I) may support the idea that cyclic GMP is of importance for lipolysis. However, Fain and Butcher (1976) failed to demonstrate any relationship between cyclic GMP and lipolysis in rat fat cells. Furthermore Fredholm (1974) found that dibutyryl cyclic GMP had minimal effects on basal or stimulated lipolysis in the isolated perfused canine ...adipose tissue. Thus the physiological significance of the present finding is obscure.

TABLE I Effect of nerve stimulation on lipolysis (glycerol release), vasoconstriction and on the cycl. AMP/cyclic GMP ratio

	Glycerol release μmol min^{-1} 100 g^{-1}	Vascular resistance PRU_{100}	Cyclic AMP / Cyclic GMP
Cont ol	0 24±0.10	21±4	5 1
During stim. 2–5 min.	0 19±0.07	95±26	6 5
During stim. 9–12 min.	0.94±0.24	60±10	17 6
After stim 2–3 min.	5 69±1 64	14±1	4.3
After stim. 4–6 min.	4 05±1.02	12±1	—
After stim. 7 11 min.	1.87±0.33	14±1	3.4

to a cyclic AMP/cyclic GMP ratio of between 1 5 and 10 We found a ratio between 3 and 1 (Table I).

Changes in cyclic nucleotide levels during nerve stimulation

The changes in cyclic nucleotide levels during nerve stimulation are summarized in Table I The increase in cyclic AMP induced by nerve stimulation is small—the average increase is 50% over basal In view of the very large increases in cyclic AMP reported following catecholamine stimulation *in vitro* (*e g* Butcher *et al* 1965 Knight and Iliffe 1973, Fredholm and Hjemdahl 1976) it may be argued that these *in vivo* changes are artefactually small. As shown in Table I the changes in glycerol release and in vascular resistance are large suggesting that the limited increase in cyclic AMP cannot be due to a failure of the electrical stimulation. On the other hand, it is possible that lipolysis is induced from a different population of cells (*e.g* cells situated deep within the tissue) than those sampled by the biopsies. It should be pointed out that the lipolytic rates observed in these expts. are close to the maximal lipolytic rates obtained in this preparation of adipose tissue by any procedure including *i.a* infusion of isoprenaline (Ballard *et al* 1971) or theophylline (Fredholm 1974) in massive doses. Therefore a selective activation of only a small number of cells seems unlikely but cannot be excluded It is of interest that the i v infusion of NA at a rate of 0.5 μg/kg/min, which produces plasma levels of NA close to 2 10^{-8} M produces a sixfold increase in lipolysis in the canine subcutaneous adipose tissue (Hjemdahl and Fredholm 1976) In isolated rat fat cells this concentration of NA produces an approximately sixfold increase in lipolysis, while alteration in cyclic AMP are undetectable (Fredholm and Hjemdahl 1976).

The possibility must thus be borne in mind that maximal or close to maximal lipolytic responses in the intact canine s.c. adipose tissue are associated with small, almost undetectable alterations in the absolute level of cyclic AMP

In some tissues, such as the liver catecholamine stimulation is followed by a very modest change in total tissue level of cyclic AMP while there is a dramatic increase in the release of cyclic AMP to the extracellular medium (Exton *et al* 1972) In order to investigate whether this is true also for adipose tissue, arterial and venous samples were analyzed for cyclic AMP The arterial concentration averages 19.8 nM (range 14 1–23 6, n = 8), while the venous was 18.2 nM (range 14 8–23.9 nM n = 8) This slight positive arteriovenous co

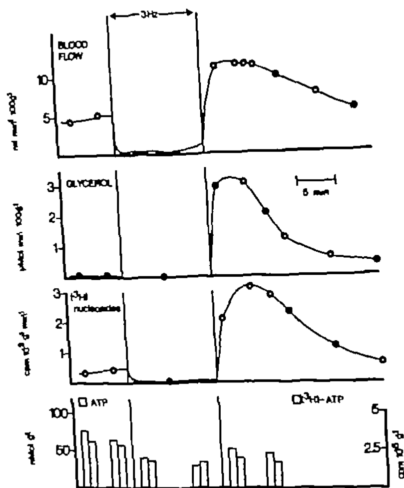


Fig. 2. Effect of sympathetic nerve stimulation (3 Hz, 12 V, 2 ms for 12 min) in canine subcutaneous adipose tissue (32 g) labelled with ^{3}H-adenine 90 min previously. For further explanation see text.

These values correspond to a "release" by adipose tissue of some 30 pmol ATP/min/g. The exact source of this ATP is not completely known, however. Thus, release from erythrocytes and platelets may be important (Forrester and Lind 1969). Furthermore, there was no change in the veno-arterial concentration difference for ATP by nerve stimulation in these experiments.

In 3 expts. the tissue adenine nucleotide stores were labelled by an infusion of ^{3}H-adenine. As seen in Fig. 1 most of the tritium is present in nucleotides, of which ATP predominates.

The results obtained in one of these expts. are illustrated in Fig. 2. The stimulation produced a marked vasoconstriction, followed by a period of hyperaemia, during which glycerol release was substantially increased. During nerve stimulation there was a parallel fall in non-radioactive and radioactive ATP. In agreement, with earlier results (Fredholm 1976) nerve

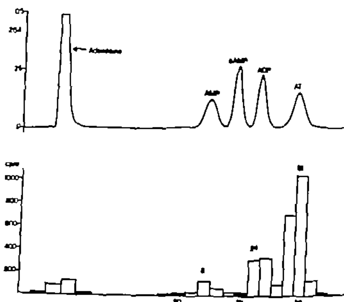


Fig. 1 Distribution of radioactivity in canine subcutaneous tissue labelled with ³H-adenine (1 100 min previously. Biopsies were cut out, frozen in liquid nitrogen, homogenized in 0.4 M perchl Protein free fat infranatant was neutralized with 4 M KOH and 1 M Trisbase. 0.6 μmol ADP A adenosine and 0.15 μmol cAMP were added as carrier and for identification of radioactiv A total volume of 1.6 ml sample was applied on top of a 0.9 22 cm column of AG1 2-200 chloride form. A gradient of HCl in water was used for elution. Absorbance was monitored on a model absorbance monitor at 254 nm with a flowcell having a light path of 0.5 cm. Fraction s

Changes in ATP levels during nerve stimulation

During nerve stimulation there was a rapid fall in tissue ATP content, which lasted fo minutes following stimulation but later the ATP content returned to control (Tabl In some expts. the ATP levels in plasma from arterial and venous blood were mea We found approximately 0.6 (n = 4) μM ATP in arterial blood and 0.9 μM (n = 8) in blood, *i.e.* levels similar to those found by Forrester and Lind (1969) in the human fo

TABLE III ATP levels in adipose tissue before, during and after nerve stimulation. Mean ± S.E within parenthesis represent umber of expts.

	ATP level i % of control			
	During stim.		After stim.	
	2–4 min[c]	7 12 min[c]	2 3 min[d]	7–12 min[d]
No drug	78 ± 12 (8) p < 0.01[b]	71 ± 6 (11) p < 0.01	73 ± 8 (9) p < 0.01	97 ± 16 (7
α-Adrenoceptor blockade[a]	100 ± 12 (6)		116 ± 17 (5)	

Control ATP levels were 73.6 ± 6.7 (21) with no drug treatment and 53.1 ± 10.9 (7) nmol/g w.w α-adrenoceptor blockade.

[a] Hydergine 250–300 μg i.a. Phentolamine 700 μg i.a.
[b] Student's t test.
[c] Refers to time after the start of stimulation.
[d] Refers to time after the end of stimulation.

[illegible], J. H., N. L. Kansky, J. G. Hardman, A. E. Broadus, E. W. Sutherland and G. W. Liddle, [illegible] of catecholamines and adrenergic blocking agents on plasma and urinary cyclic nucleotides in [illegible] *J. clin. Invest.* 1972, *51* 2124–2129

[illegible], K. C. A. Coss and S. Rosell, Vascular and lipolytic responses in canine subcutaneous adipose tissue following infusion of catecholamines. *Acta physiol. scand.* 1971 *81* 246–253.

[illegible], L and B. Jeanrenaud, ATP content of isolated fat cells. Effects of insulin, ouabain, and lipolytic [illegible]. *Biochim. biophys. Acta* (Amst.) 1970. *202* 496–506.

[illegible], B. L., R. P. Ekins and J. D. M. Albano, Saturation assay for cyclic AMP using endogenous binding protein. *Adv. cyclic Nucl. Res.* 1972. *2*, 25–40.

[illegible], R. W., R. J. Ho, H. C. Meng and E. W. Sutherland, Adenosine 3',5'-monophosphate in biological materials. II. The measurement of adenosine 3',5'-monophosphate in tissues and the role of the cyclic nucleotide in the lipolytic response of fat to epinephrine. *J. biol. Chem.* 1965. *240*, 4515–4523

[illegible], R. M., R. E. Young and P. J. Randle, Measurement of concentrations of metabolites in adipose tissue and effects of insulin, alloxan-diabetes and adrenaline. *Biochem. J.* 1966. *100*, 407–419.

[illegible], J. H., S. B. Lewis, R. J. Ho and C. R. Park, The role of cyclic AMP in the control of hepatic glucose production by glucagon and insulin. *Adv. cyclic Nucl. Res.* 1972. *1* 91–120.

[illegible], J. N., Biochemical aspects of drug and hormone action on adipose tissue. *Pharmacol. Rev.* 1973. *25* [illegible]–113

[illegible], J. N. and F. R. Butcher, Cyclic guanosine 3',5'-monophosphate and the regulation of lipolysis in [illegible] fat cells. *J. cyclic Nucl. Res.* 1976. *2* 71–78.

[illegible], L. O., B. B. Fredholm, B. Hamberger, P. Hedqvist and L. Westman, Plasma cyclic AMP and metabolic substrates following hemorrhagic shock in the rat. *Acta chir. scand.* 1977. In press.

[illegible], G. P. Doggio, R. Badetti and L. Vireo, Effect of oxidative phosphorylation inhibitors on cyclic adenosine monophosphate synthesis in rat adipose tissue. *Biochem. Pharmacol.* 1972 a. *21* 1633–1639

[illegible], G. P. Doggio, G. Pirini and E. Tóth, Effect of glycolysis inhibitors on cyclic AMP synthesis [illegible] rat adipose tissue. *Biochem. Pharmacol.* 1972 b. *21* 2295–2301

[illegible], T. and A. R. Lind, Identification of adenosine triphosphate in human plasma and the concentration in the venous effluent of forearm muscles before, during and after sustained contractions. *J. Physiol.* (Lond.) 1969. *204*, 347–364

[illegible], B. B. Studies on the sympathetic regulation of circulation and metabolism in canine subcutaneous adipose tissue. *Acta physiol. scand.* 1970. *80* Suppl. 354. 1–47

[illegible], B. B. Vascular and metabolic effects of theophylline, dibutyryl cyclic AMP and dibutyryl cyclic GMP in canine subcutaneous adipose tissue in situ. *Acta physiol. scand.* 1974. *90*, 226–236.

[illegible], B. B. Release of adenosine-like material from isolated perfused dog adipose tissue following sympathetic nerve stimulation and its inhibition by adrenergic α-receptor blockade. *Acta physiol. scand.* 1976. *96* 422–430

[illegible], B. B. and A. F-----, High energy phosphate compounds in adipose tissue: The effect of hemorrhage. *Acta physiol. scand.* 1974. *91* 165–171

F-----, B. B. and P. Hedqvist, Inhibition by acidosis of adenosine 3',5'-cyclic monophosphate accumulation and lipolysis in isolated rat fat cells. *Acta physiol. scand.* 1976. *96* 160–169

Fredholm, B. B. and J. Karlsson, Metabolic effects of prolonged sympathetic nerve stimulation in canine subcutaneous adipose tissue. *Acta physiol. scand.* 1970. *80*. 567–576.

Fredholm, B. B., B. Linde, R. L. Prewitt and P. C. Johnson, Oxygen uptake and tissue oxygen tension during adrenergic stimulation in canine subcutaneous adipose tissue. *Acta physiol. scand.* 1976. *97* 48–59

Fredholm, B. B. and S. Rosell, Effects of adrenergic blocking agents on lipid mobilization from canine subcutaneous adipose tissue after sympathetic nerve stimulation. *J. Pharmacol. exp. Ther.* 1968. *159* 1–7

Goldberg, N. D., M. K. Haddox, S. E. Nicol, D. B. Glass, C. H. Sanford, F. A. Kuehl, Jr. and R. Estensen, Biological regulation through opposing influences of cyclic GMP and cyclic AMP: The Yin Yang hypothesis. *Adv. cyclic Nucl. Res.* Vol. 5. (Eds. G. I. Drummond, P. Greengard and G. A. Robison). Raven Press, New York 1975. 307–330.

Hansen, R. W. and F. J. Ballard, The concentrations of metabolites of glucose in rat adipose tissue cells in vitro. *Biochim. biophys. Acta* (Amst.) 1970. *201* 196–204.

Harper, J. F. and G. Brooker, Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'O-acetylation by acetic anhydride in aqueous solution. *J. cyclic Nucl. Res.* 1975. *1* 207–218

stimulation was associated with a marked increase in the release of radioactive (and nucleoside) material Based on the specific activity of the tissue ATP (4.3 ± 10^{6} cpm/nmol) it can be calculated that totally about 0 8 nmol purine material was released per g tissue. This corresponds to only 1 % of the total ATP content. Thus release of ATP per se or in the form of nucleosides cannot be held responsible for the fall in ATP during nerve stimulation.

Furthermore, the fall in ATP cannot be accounted for by a rise in cyclic AMP since it was three orders of magnitude less. Thus, the most reasonable explanation is that nerve stimulation induces a shift in the nucleotide pool from ATP to less energy-rich compounds. Support for this contention is afforded by preliminary findings that ³H-nucleotides, separated as described in Fig. 1 in biopsies taken immediately following nerve stimulation were shifted towards ADP and AMP

As noted in the Introduction a fall in ATP levels has been found also in fat cells and adipose tissue *in vitro* This seems to be due, at least in part, to the accumulation of intracellular fatty acids, which may inhibit ATP synthesis (Angel *et al* 1971) The fall seen in the present study appears to be more rapid than that observed *in vitro* It is therefore possible that it has a different explanation It was recently found that adrenergic stimulation causes a marked fall in tissue oxygen tension (Fredholm *et al* 1976). This seems to be due to the combined effect of a decreased gross blood flow a decreased number of open capillaries and a stimulated metabolism, or in other words due to both α-adrenoceptor actions on the vasculature and β-adrenoceptor actions on metabolism We therefore carried out some expts. in tissue treated with α-adrenoceptor blocking agents, which eliminate the vasoconstrictor effect of nerve stimulation. As seen in Table III the effect on ATP levels were also abolished. This supports the idea that the shift in the adenine nucleotide pool seen as a fall in ATP in adipose tissue may be due to local hypoxia secondary to decreased blood perfusion and decreased number of open capillaries.

Under the present experimental conditions the fall in ATP was reversible. However if hypoxia is prolonged, *e.g* in hemorrhagic shock there is an irreversible fall in ATP levels (Fredholm and Fronek 1974) There are several possible explanations for this irreversibility. First, there may be irreversible damage to the ATP synthesizing machinery in the cells, for example to the mitochondria. Secondly the release of purine compounds may if prolonged sufficiently lead to a depletion of substrate for ATP synthesis. Since the ATP depletion of cells in certain target organs during shock and other traumatic conditions probably accounts for an important part of irreversible tissue damage, it is of considerable therapeutic interest to find out the mechanisms involved. The present results suggest that adipose tissue may be a useful organ to pursue such studies in.

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References

ANGEL, A., K. DESAI and M L. HALPERIN, Free fatty acid and ATP levels in adipocytes during lipolysis. *Metabolism* 1971 *20* 87 99

ARNER, P and J ÖSTMAN, Methodological aspects of protein-binding assays for cyclic AMP in human adipose tissue. *Scand. J clin. Lab. Invest* 1975 *35* 691–697

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Renal Na-K ATPase Activity during Saline Infusion in the Rabbit

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Abstract

SEJERSTED O. M. *Renal Na-K ATPase activity during saline infusion in the rabbit.* Acta physiol. scand. 1977 99 323–335

During saline infusion, sodium reabsorption (R_{Na}) in the diluting segment (thick ascending limb of Henle's loop) increases acutely. The mechanism for this higher pumping rate of outer medullary Na-K ATPase is unknown. Following left-sided nephrectomy, immediate [illegible] infusion of hypertonic saline increased R_{Na} in the remaining whole right kidney by 28 ± 14 [illegible] (p [illegible] 0.05). Na-K-ATPase activity in outer medulla was raised by (Δ) 23 [illegible] 4 [illegible] above the left kidney (p [illegible] 0.05), whereas cortical activity was unchanged. The mechanism for this increase in Na-K-ATPase activity was explored. The catalytic rate per enzyme did not differ in the two kidneys and equalled 5 340 min^{-1}. The increase was therefore due to higher tissue concentration of active enzyme. The response was fully developed during continuous infusion within 20 min, and of equal magnitude [illegible] protein synthesis had been inhibited by cycloheximide (Δ [illegible] 23 ± 7 [illegible]) or stimulated by unilateral nephrectomy 6 days earlier combined with saline infusion for 2 h (Δ = 34 ± 10 [illegible]). Thus, during hypertonic saline infusion, the increased R_{Na} in the outer medulla was partly accounted for by the activation of latent Na-K-ATPase. High delivery of sodium to the diluting segment for more than [illegible] during hypertrophy caused no further activity change.

Key words: Active sodium reabsorption, cycloheximide, enzyme activation, ion transport, kidney medulla, loop of Henle, renal hypertrophy

Saline infusion increases both the sodium reabsorption in the thick ascending limb of Henle's loop (diluting segment) (Rector *et al.* 1964, Schnermann 1968, Kunau *et al.* 1974) and the metabolic rate of the outer medulla (Kill *et al.* 1971). Several reports suggest that sodium reabsorption is carried out in this segment by Na-K ATPase (Katz and Epstein 1967, Nechay and Nelson 1970), located at the peritubular cell membrane (Wachstein and Besen 1964, Kyte 1976). Moreover because inhibition of Na-K ATPase with cardiac glycosides greatly reduces both sodium reabsorption in the diluting segment (Martinez-Maldonado *et al.* 1970) and the metabolic rate of the outer medulla (Sejersted *et al.* 1971, Lie *et al.* 1974), it is therefore possible that renal Na-K-ATPase can be stimulated by saline infusion.

Kill (1971) proposed that sodium reabsorption in the diluting segment is raised during increased distal delivery of sodium because a rise in intracellular sodium concentration

HEPP D D R. CHALLONER and R. H WILLIAMS, Studies on the action of [illegible] in isolated [illegible] tissue cells. I Stimulation of incorporation of [32]P-labelled inorganic phosphate into [illegible] in the absence of glucose. *J biol. Chem* 1968. *243* 4020–4026.

HJEMDAHL, P and B. B. FREDHOLM, Influence of adipose tissue blood flow on the lipolytic [illegible] circulating noradrenaline at normal and reduced pH. *Acta physiol scand.* 1976. *98* 74–79

HOLLENBERGER, C. H and R. L. PATTEN, Relation of fat cell ATP content to lipolysis induced [illegible] dibutyryl 3 ,5 -cyclic AMP *Metabolism* 1970. *19* 856–864

HUTTUNEN, J K., D STEINBERG and S. E. MAYER, ATP-dependent and cyclic AMP-independent [illegible] tion of rat adipose tissue lipase by protein kinase from rabbit skeletal muscle. *Proc nat Acad Sci* (Wash) 1970 *67* 290–295

ILLIANO, G G P TELL, M I SIEGEL and P CUATRECASAS, Guanosine 3 ,5 -monophosphate and the action of insulin and acetylcholine. *Proc nat Acad Sci* (Wash.) 1973 *70* 2443–2447

ISSEKUTZ, T B., Estimation of cyclic AMP turnover in normal and methyl-prednisolone-treated [illegible] effect of catecholamines. *Amer J Physiol* 1975 *229* 291–297

KASHYAP M L., J S L. TAY S P SATHY and J A. MORRISON, Role of adipose tissue in free fatty [illegible] metabolism in hemorrhagic hypotension and shock *Metabolism* 1975. *24* 855–860.

KNIGHT B. L. and J ILIFFE, The effect of glucose, insulin and noradrenaline on lipolysis, and on [illegible] concentrations of adenosine 3 5 -cyclic monophosphate and adenosine 5 triphosphate in [illegible] tissue. *Biochem. J* 1973 *132* 77–82.

LAURELL, S and G TIBBLING, An enzymatic fluorimetric micromethod for the determination [illegible] glycerol. *Clin. chim. Acta* 1966. *13* 317–322.

NGAI, S. H S. ROSELL and L. WALLENBERG, Nervous regulation of blood flow in the subcutaneous adipose tissue in dogs. *Act physiol scand* 1966. *68* 397–403

ROSELL, S Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic [illegible] stimulation. *Act physiol scand.* 1966. *67* 343–351

STANLEY P E. and S. E. WILLIAMS, Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Analyt Biochem.* 1969 *29* 381 392.

STEINER, A. L., C. W PARKER and D M KIPNIS, Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides. *J biol Chem.* 1972. *247* 1106–1113.

WIENER, S., R. WIENER, M URIVETZKY and E. MEILMAN Coprecipitation [illegible] ATP with potassium perchlorate. The effect on the firefly enzyme assay of ATP in tissue and blood. *Analyt Biochem* [illegible] *59* 489–500.

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...ng saline infusion, sodium reabsorption (R_{Na}) in the diluting segment (thick ascending limb of Henle ...) increases acutely. The mechanism for this higher pumping rate of outer medullary Na-K ATPase ...known. Following left-sided nephrectomy immediate infusion of hypertonic saline increased ... in the remaining whole right kidney by 28 ± 14 (p 0.05). Na-K ATPase activity in outer medulla ...rased by (Δ) 23 ± 4 above the left kidney (p 0.05), whereas cortical activity was unchanged. The ...chanism for this increase in Na K-ATPase activity was explored. The catalytic rate per enzyme did not ... in the two kidneys and equalled 5 340 min^{-1}. The increase was therefore due to higher tissue con...tration of active enzyme. The response was fully developed during continuous infusion within 20 min, ... of equal magnitude whether protein synthesis had been inhibited by cycloheximide (Δ = 23 ± 7) ...imulated by unilateral nephrectomy 6 days earlier combined with saline infusion for 2 h (Δ 34 ± 10 ...). ...s, during hypertonic saline infusion, the increased R_{Na} in the outer medulla was partly accounted for ... the activation of latent Na-K ATPase. High delivery of sodium to the diluting segment for more than ... during hypertrophy caused no further activity change.

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...line infusion increases both the sodium reabsorption in the thick ascending limb of Henle loop (diluting segment) (Rector *et al.* 1964, Schnermann 1968, Kunau *et al.* 1974) and the metabolic rate of the outer medulla (Kiil *et al.* 1971). Several reports suggest that sodium reabsorption is carried out in this segment by Na-K-ATPase (Katz and Epstein 1967, Nechay and Nelson 1970), located at the peritubular cell membrane (Wachstein and Meisel 1964, Kyte 1976). Moreover because inhibition of Na-K-ATPase with cardiac glycosides greatly reduces both sodium reabsorption in the diluting segment (Martinez Maldonado *et al.* 1970) and the metabolic rate of the outer medulla (Sejersted *et al.* 1971, Lie *et al.* 1974). It is therefore possible that renal Na-K-ATPase can be stimulated by saline infusion.

Kiil (1971) proposed that sodium reabsorption in the diluting segment is raised during increased distal delivery of sodium because a rise in intracellular sodium concentration

would stimulate Na K ATPase. In addition to such direct substrate dependent stimulation the pumping rate could be increased due to higher catalytic rate for each enzyme (turnover number) even at constant sodium concentration *or* by a rise in the tissue enzyme concentration.

This study shows that in anesthetized rabbits the turnover number for renal Na-K-ATPase remained unchanged but that the concentration of active enzyme in the outer medulla increased by 23 % during hypertonic saline infusion. This increment might be due either to synthesis of new enzyme protein or to recruitment of inactive enzyme. Experiments were therefore carried out to see whether the stimulation of Na-K-ATPase activity occurred too rapidly to be accounted for by protein synthesis or whether the response was affected by inhibition of protein synthesis with cycloheximide.

It is well known that renal Na K ATPase activity increases after nephrectomy but the effect could be due to specific stimulation beyond the general stimulation of protein synthesis which can be attributed to hypertrophy (Katz and Epstein 1967). Hence, the effect of combination of unilateral nephrectomy and infusion of saline for 2 h 6 days later was compared to the increase in activity obtained after 20 min infusion.

The increment in Na K ATPase activity in outer medulla during hypertonic saline infusion probably does *not fully* account for the rise in sodium reabsorption in the diluting segment but might be important in amplifying the effect of a rise in substrate concentration, i.e. the sodium concentration at the intracellular side of the peritubular membrane (Kill 197

Methods

Rabbits of both sexes weighing 1.5–3 2 kg were kept on a standard diet containi g about 60 μmol sodium and ≃50 μmol potassium per gram. They were fasted overnight but had free access to ater Anesthesia was induced by sodium pentobarbital 30 mg/kg b.wt. (Nembutal), and mai tained by additional doses of 5 mg/kg b wt. dministered through Venflon into an ear vein. Complete analgesia as obtained by subcutaneous jection of 20–30 mg lidocain chlorid (Xylocain) before incisions were made. Free airways were ensured by tracheostomy and body temperatur mai tained with a heating lamp.

When cleara ce was determined, polyethylene catheters were inserted i t a carotid artery and a jugular vein. The arterial catheter served fo blood sampling and the pressure was recorded with Statham pressure transducer (*P35Gb*) and a *Sanborn* mplifier nd a recorder All infusions were kept at body temperature. Both ureters were exposed thro gh a midline bdominal incison and cannulated with soft polyvinyl catheters. The incision was closed by towel clips.

Experimental procedure

The clearance of 51Cr EDTA was determined as an estimate of glomerula filtration rate (GFR) 35 μCi was injected i.v followed by a continuous infusio of 0.45 % NaCl and 2.5 % glucose at a rate of 22 μl min co taining 30 μCi 51Cr EDTA per ml, thus obtaind g arterial concentrations of 3 000–6 000 cpm/ml. At least one hour elapsed before rine collection was started

Acute experiments were performed n four groups of rabbits. In 6 rabbits which served as control group (Group I, Control), no clearance was determined Through the abdominal incision the two kidneys were removed with a 20 min interval for enzyme analysis. I the second group (Group II, Acute Experiment, 7 rabbits), two control periods, with urine sampling for 30–60 min, elapsed before removal of the left kidney Subsequently 13 1 ± 0.5 mmol/kg b.wt. sodium chlorid in 1 45 % sol tion (52 ml/kg b t) was infused i v Half of the amou t of saline was given in the course of 2 3 min, the rest at rate of 3.5 ml/min When three 1 min urine samples had been btained, the right kidney was removed exactly 20 min after the left.

T test the effect of protein synthesis inhibition, the response to hypertonic saline was measured followed

[illegible] I. Effect of cycloheximide on the incorporation of 3H leucine into renal microsomes.

	Cortex dpm/mg prot.	Outer medulla dpm/mg prot.
[illegible]trol	7 980	6 630
[illegible]	±440	±440
[illegible]heximide		
[illegible] mg/kg body wt)	990	1 960
[illegible]	±130	±80
	0.05	<0.05

[illegible] had been unilaterally nephrectomized 4 days prior to the experiment. Following injection [illegible] cycloheximide or saline in the control group 3H-leucine, 120 μCi/kg b wt. was injected [illegible] 3 h later the [illegible] kidney was removed and analyzed. Mean values ± standard error of the mean.

[illegible]heximide 5 mg/kg b.wt. (Group III, Cycloheximide and Acute Expansion, 7 rabbits). The drug was [illegible] following 2 control periods. Urine [illegible] collected for another 2 periods before the removal [illegible] the left kidney. Saline was infused as described above and right-sided nephrectomy performed after 20 [illegible]

For control, the effect of cycloheximide on the rate of 3H-leucine incorporation in renal microsomes [illegible] tested on 10 separate rabbits (Group IV Cycloheximide). Unilateral nephrectomy had been performed [illegible] days earlier to stimulate protein synthesis. The drug was injected [illegible] in 5 rabbits and saline [illegible] in [illegible] others [illegible] before 120 μCi/kg b.wt. 3H-leucine was administered [illegible] The kidney was removed 3 [illegible] and radioactivity measured in the cortex and outer medulla. Microsomal tissue, prepared as described, [illegible] dissolved in 2 ml Soluene 350 (Packard) and added to 10 ml Dimilume (Packard) in counting [illegible] [illegible] incorporation was reduced by 88 [illegible] in cortical tissue and 75 [illegible] in outer medulla (Table I).

The effect of 6 days hypertrophy following left-sided nephrectomy combined [illegible] with saline expansion for [illegible] h [illegible] tested in 5 rabbits (Group V Nephrectomy and Prolonged Expansion). Following the hypertrophy [illegible], 45 mmol sodium chloride [illegible] administered: 8–9 mmol as [illegible] 1.45 [illegible] solution (32–36 ml) in the course [illegible] 12–3 min, the rest as [illegible] continuous isotonic [illegible] infusion. 51Cr EDTA clearance was measured only in [illegible] right kidney before and during the infusion. Enzyme activities were compared in the left and right kidneys.

The 3H activity [illegible] measured in an Intertechnique SL 30 liquid scintillation spectrometer and 51Cr [illegible] EDTA activity in [illegible] Selektronik [illegible] scintillation counter. Sodium and potassium were measured by [illegible] flame photometer using lithium as the internal standard (Instrumentation Laboratories 343).

Tissue preparation and enzyme analysis

Following removal, the kidneys were gently compressed and thereby drained for blood and urine before [illegible] in ice-cold buffer containing 30 mmol/l histidine and 250 mmol/l sucrose [illegible] pH of 7.2 [illegible] 20 C (histidine-sucrose buffer). During the following procedures the tissue was kept at 0°C.

Tissue from cortex and outer medulla [illegible] homogenized separately in histidine-sucrose buffer ([illegible] [illegible]) [illegible] Potter-Elvehjem Teflon/glass homogenizer. Crude homogenate was obtained by extra homogenization of 2 ml of the suspension in [illegible] small glass tube with [illegible] wall/pestle clearing of 0.11–0.15 mm. The microsomal fraction was prepared as described by Jørgensen and Skou (1971). Following centrifugation (90 [illegible] 10^3 g [illegible] min) the pellet was resuspended in buffer, homogenized, and centrifuged anew. The combined supernatants were then spun for 1.5 [illegible] 10^6 g [illegible] min, the pellet suspended in buffer and regarded as the microsomal fraction. These enzyme preparations could be stored frozen for weeks without loss of Na-K ATPase activity. However, during preparation of microsomes, more than 90 [illegible] of the tissue protein was discarded and of the total enzyme activity in the crude homogenate, [illegible] variable fraction of about [illegible] 5 [illegible] recovered. As [illegible] consequence, there is poor correlation between activities measured in crude homogenate and microsomes (Fig. 1). In this study enzyme activity [illegible] therefore measured as rate of ATP hydrolysis per mg protein in the crude homogenate.

Enzyme activity was measured simultaneously in the two kidneys from the same rabbit by the method described by Jørgensen and Skou (1971). The enzyme was preincubated in 25 imidazol, 2 EDTA, 1.7 Na-deoxycholate (NaDOC) (mmol/l), pH 7.0 for 30 min at 20°C. This concentration of NaDOC produced the highest enzyme activity. Of the crude homogenate, 0.5–1.0 mg protein [illegible] preincubated in [illegible] volume of

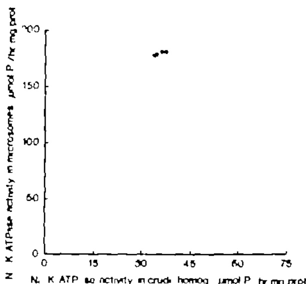


Fig. 1 Comparison of Na-K-ATPase activity in crude homogenate and microsomes from outer medulla in 31 kidneys. The correlation is significant (r=0.4 0.005 < p < 0.01) but only 19 of the variation in microsomal activity is explained by the variation in homogenate activity

1 ml and similarly 10–40 μg protein from the microsomes in a volume of 0.1 ml. 25 μl of the preparation was transferred to 1 ml of the incubation medium containing 30 histidine, 3 Tris-ATP, 3 $MgCl_2$, 100 20 KCl, 1 phosphoenolpyruvate (PEP) (mmol/l) and 30 μg pyruvate kinase (PK). The reaction was stopped after 10 min at 37°C with 0.1 ml 50 trichloroacetic acid (TCA). Measurements were taken in duplicate and Na K ATPase activity calculated on the basis of the difference in ATP hydrolysis rate in the absence and presence of ouabain, 1 mmol/l. Data for Mg-ATPase are not included because this enzymatic activity is unstable on preparation and storage (unpublished observation).

Inorganic phosphate was determined according to Fiske and SubbaRow (1925) using AMIDOL (BDH Chemicals Ltd.) as a reducing agent. Protein was determined in the enzyme preparations with the Folin reagent (Merck) ad modum Lowry *et al* (1951). A standard curve was obtained by serial dilution of enzyme preparation where the protein content had been measured by the Kjeldahl nitrogen method. Bovine serum albumin (Sigma Chemical Company) dissolved in histidine-sucrose buffer was used as standard. Photometric readings were carried out in a Beckman DU spectrophotometer

The ionic concentrations in the incubation medium were chosen to give maximal enzyme activity (Fig. 2). Tris ATP was prepared from the disodium salt of "Sigma grade" ATP (Sigma Chemical Company

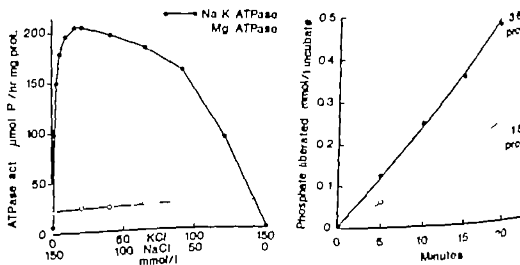
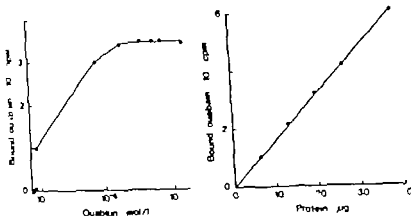


Fig. Incubation characteristics of outer medullary microsomes. Left. Effect of alterations in sodium potassium concentration. Right. Reaction rate is linear and independent of the protein concentration



1. Binding of 3H-ouabain to microsomal Na-K-ATPase from outer medulla. Left. Effect of varying the ouabain concentration. Right: Standard curve with ouabain concentration 4 10^{-4} mol/l.

an ion exchange column (Amberlite Resin IR 120, Standard grade, BDH Chemicals Ltd) Less than inhibitory effect of this preparation could be observed in contrast to the great inhibition reported the disodium salt (Charney et al. 1975). Apparent K_m for Tris ATP was found to be less than 0.5 ol/l

rnover rate was constant even at concentrations of split phosphate reaching 20 of the original ATP entration (Fig 2) hich was kept constant by the PEP/PK system.

ouabain saturation binding

ding of ouabain to microsomes from outer medulla was used as an estimate of enzyme concentration. ording to Jørgensen (1974), the number of ouabain binding sites equals the number of ATP binding s and therefore assumed binding of one mole ouabain to one mole enzyme. Enzyme activity per bain binding site is then an estimate of the turnover number

The saturation technique was preferred because much more tissue was required to obtain acceptable tchard plots, more than could be obtained from the entire outer medulla of one rabbit kidney

Following preincubation as described, 100 µl (50–150 µg protein) ere transferred to tubes containing 10^{-4} 3H-ouabain, 30 Tris-HCl, 3 Tris-ATP 5 $MgCl_2$, 120 NaCl (mmol/l) in final volume of 1 ml h pH 7.4 at 25°C, and incubated for 2 h at 37°C. Unspecific binding was measured following incubation th only 3H-ouabain and Tris-HCl The incubate was then filtered by means of Millipore filters (GSWP 500), washed ith 15 ml ice-cold Tris-HCl 30 mmol/l, dried at 80°C, and counted in vials containing 10 l toluene ith 5 g PPO and 0.05 g POPOP per litre.

Filters with pore size 0.22 µm ere found to retain all measurable Na-K ATPase activity Binding of abain was maximal and stable after 60 min incubation Binding was dependent on the $MgCl_2$ concentration, but saturated at concentrations above 3 mmol/l. As evident from Fig. 3, maximal binding was obtained th ouabain concentrations abov 2.0 10^{-4} mmol/l and 4 10^{-4} mmol/l as chosen as the standard concentration. The linearity of the standard curve (Fig. 3) indicates that this concentration was adequate for maximal binding ithin the range of protein indicated. The concentration is at least 100 times higher than he maximal concentration of ouabain binding sites. Consequently less than 1 % of the ouabain was bound. Assuming equilibrium kinetics, can be calculated that even with dissociation constant of about 10 mol l, hich is much higher than reported in other studies (Hegyvary 1976), more than 98 % of the ouabain binding sites would be occupied.

3H-ouabain (Amersham) was purified by extraction with microsomes from dog renal outer medulla (about 80 of the activity was extractable) and diluted with unlabelled ouabain (Merck) to give 30 50 cpm nmol (about 0.34 Ci/mmol) when counted bound to enzyme on the filters. With this counting system quenching was constant.

Crude homogenate could not be used for binding measurements because the filters got jammed. A identical incubation conditions, the turnover number of the enzyme population is assumed homogeneous and the microsomal Na-K ATPase therefore representative.

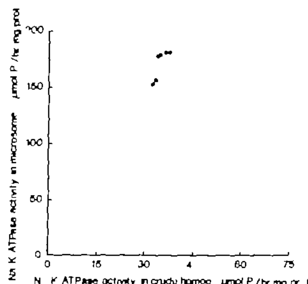


Fig. 1 Comparison of Na-K-ATPase activity in crude homogenate and microsomes from outer medulla in 30 tubes. The correlation is significant (r=0.4, $0.005 < p < 0.01$) but only 15% of the variation in microsomal activity is explained by the variation in homogenate activity

1 ml and similarly 10–40 µg protein from the microsomes in volume of 0.1 ml. 25 µl of the preparation was transferred to 1 ml of the incubation medium containing 30 histidine, 3 Tris-ATP, $3MgCl_2$, 130 Na, 20 KCl, 1 phosphoenolpyruvate (PEP) (mmol/l) and 30 µg pyruvate kinase (PK). The reaction was stopped after 10 min at 37°C with 0.1 ml 50% trichloroacetic acid (TCA). Measurements were taken in duplicate and Na K ATPase activity calculated on the basis of the difference in ATP hydrolysis rate in the absence and presence of ouabain, 1 mmol/l. Data for Mg-ATPase are not included because this enzymatic activity is unstable on preparation and storage (unpublished observation).

Inorganic phosphate was determined according to Fiske and SubbaRow (1925) using AMIDOL (BDH Chemicals Ltd.) as reducing agent. Protein was determined in the enzyme preparations with the Folin reagent (Merck) ad modum Lowry *et al.* (1951). A standard curve was obtained by serial dilution of an enzyme preparation where the protein content had been measured by the Kjeldahl nitrogen method. Bovine serum albumin (Sigma Chemical Company) dissolved in histidine-sucrose buffer was used as a routine standard. Photometric readings were carried out in Beckman DU spectrophotometer.

The ionic concentrations in the incubation medium were chosen to give maximal enzyme activity (Fig. 2). Tris-ATP was prepared from the disodium salt of "Sigma grade" ATP (Sigma Chemical Company

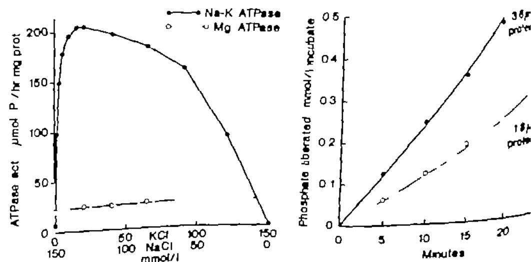


Fig. 2. Incubation characteristics of outer medullary microsomes. Left. Effect of alterations in sodrum and potassium concentration. Right. Reaction rate is linear and independent of the protein concentration

Table III. Effect of 1.45% saline infusion iv on blood pressure, glomerular filtration rate, sodium excretion and reabsorption, and potassium excretion in Groups II, III and V

	BP mmHg	GFR ml/min		P_{Na} mmol/l	$U_{Na}V$ μmol/min		Na reab μmol/ml		P_K mmol/l	U_KV μmol/min	
		left kidney	right kidney		left kidney	right kidney	left kidney	right kidney		left kidney	right kidney
Group II											
Control	76 ±4	2.9 ±0.5	3.1 ±0.6	142 ±2	1.9 ±0.6	1.8 ± 0.7	410 ± 70	438 ± 80	2.2 ±0.1	1.9 ±0.3	-0 ±0.3
Acute Expansion	84 ±6		6.6 ±0.6	154 ±2		453 ±73		542 ± 53	1.8 ±0.1		8.1 ±1.4
Group III											
Control	79 ±6	4.0 ±0.5	3.8 ±0.5	144 ±1	4.0 ±1.6	3.8 ± 1.6	572 ± 77	542 ± 77	2.3 ±0.2	2.7 ±0.3	2.6 ±0.4
Cyclo-heximide	80 ±7	4.8 ±1.0	4.3 ±0.7	143 ±2	3.4 ±1.4	3.0 ± 1.1	687 ±138	622 ±105	-6 ±0.3	-7 ±0.5	2.5 ±0.4
Cyclo-heximide Acute Expansion	91 ±7		7.2 ±0.8	158 ±2		495 ±62		639 ± 82	2.1 ±0.2		9.4 ±1.6
Group V											
Nephrectomy	76 ±8		4.7 ±1.2	146 ±2		3.0 ± 0.7		682 ±167	2.9 ±0.2		4.0 ±0.6
Nephrectomy Prolonged Expansion	98 ±7		7.5 ±0.8	158 ±2		228 102		925 ±123	2.6 ±0.2		7.8 ±0.3

BP = blood pressure. GFR = glomerular filtration rate. $U_{Na}V$ = sodium excretion. U_KV = potassium excretion, P_{Na} = plasma sodium concentration. P_K = plasma potassium concentration. Mean values ± standard error of the mean.

The response of outer medullary Na-K-ATPase activity to hypertonic saline infusion was unaffected after inhibition of the renal protein synthesis (cycloheximide, 5 mg/kg b.wt., Table II, Group III) with 23±7% increase in enzyme activity. In this group, GFR was raised by 67% and fractional sodium reabsorption lowered to 56±3%. Sodium reabsorption was not significantly changed, but the natriuretic response was similar in the group with intact protein synthesis (Table III).

Saline infusion might possibly change the sensitivity of renal tissue to NaDOC. In Fig. 4 it is shown that the enzyme activity in homogenate from outer medulla was 30±3% (SD) higher in the left kidney at all concentrations of NaDOC.

Effect of nephrectomy and prolonged saline expansion (*Group V*)

During a postnephrectomy period of 6 days, the dry weight of the left kidney increased 17 4% above the right. Combined with hypertonic saline infusion for 2 h the hypertrophy raised outer medullary Na-K-ATPase activity by 34±10%, not significantly more than during acute saline expansion (Table II). Cortical activity remained unchanged.

TABLE II Effect of acute and prolonged saline expansion on Na K ATPase activity in the crude homogenate from rabbit kidneys.

	Cortex				Outer medulla		
	Na K ATPase act., μmol P_i/hr mg prot.		Ratio right/left	p	Na K ATPase act., μmol P_i/hr·mg prot.		Ratio right/left
	left kidney	right kidney			left kidney	right kidney	
Group I Control (n=6)	22.3 ±1 5	23 6 ±1 6	1.06 ±0.03		32.3 ±1.3	32.9 ±3.3	1.02 ±0.10
p	=0 05				NS		
Group II Acute Expansion (n=7)	25.2 ±2.1	27.3 ±2.0	1.08 ±0.04	NS	27.3 ±2.6	33.6 ±2.8	1.[illegible] ±0.04
p	=0.05				<0.05		
Group III Cycloheximide and Acute Expansion (n=7)	27.8 ±1 4	29.3 ±1.3	1 05 ±0.06	NS	29.9 ±1 7	36.7 ±1.5	1.[illegible] ±0 07
p	NS				<0.05		
Group V Nephrectomy and Prolonged Expansion (n=5)	27 9 ±2.4	31 7 ±1.5	1 14 ±0 09	NS	45 6 ±3 4	61.2 ±[illegible]9	1 34 ±0.10
p	NS				=0 05		

Left kidney: control right kidney: experimental. Mean values ± standard error of the mean. p: Wilcoxon signed rank test for paired comparisons. p: p-values for comparison with the ratios of the control (Group I) with Wilcoxon signed rank test.

Statistics

All values are given as means ± standard errors of the means (S.E.), when not otherwise stated; ratios were calculated according to Cameron (1960)

Wilcoxon signed rank test for paired comparisons was used to evaluate differences between left kidneys, whereas groups were compared with the ordinary Wilcoxon signed rank test.

Regression was calculated by the method of least squares and probability of correlation by c tested by t statistics (Hodges and Lehmann 1970)

Results

Effect of i.v. infusion of 1 45% saline for 20 min

During control conditions, Na K ATPase activity for the whole kidney averaged μmol P_i/g min and the sodium reabsorption, 53 ± 8 μmol/g min (wet kidney). Following hypertonic saline infusion, Na K ATPase activity was not significantly in cortex but was higher by 23 ± 4% in outer medulla (Table II group II) The hy infusion raised plasma sodium concentration by 12 mmol/l, doubled GFR, and to sodium reabsorption increased by 28 ± 14% ($p < 0.05$) (Table III). Fractional reab of sodium fell to 57 ± 5%, whereas the fractional excretion of potassium increa 30 ± 3% to 71 ± 11% despite a fall in plasma potassium concentration (Table III).

Table III. Effect of 1.45% saline infusion iv on blood pressure, glomerular filtration rate, sodium excretion and reabsorption, and potassium excretion in Groups II, III and V

	BP mmHg	GFR ml/min		P_{Na} mmol/l	$U_{Na}V$ µmol/min		Na reab µmol/min		P_K mmol/l	U_KV µmol/min	
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BP blood pressure, GFR glomerular filtration rate, $U_{Na}V$ – sodium excretion, U_KV – potassium excretion, P_{Na} plasma sodium concentration, P_K – plasma potassium concentration. Mean values ± standard error of the mean.

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During a postnephrectomy period of 6 days, the dry weight of the left kidney increased 17 4% above the right. Combined with hypertonic saline infusion for 2 h the hypertrophy raised outer medullary Na-K-ATPase activity by 34±10%, not significantly more than during acute saline expansion (Table II). Cortical activity remained unchanged.

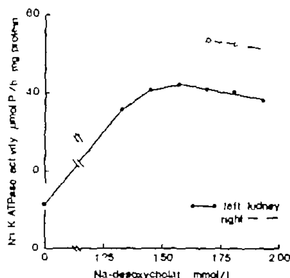


Fig. 4 Comparison of the effect of pretreatment with Na-desoxycholate on outer medullary homogenate from the left (control) and the (hypertonic saline infusion for 20 min) in one rabbit.

In the hypertrophied kidney sodium reabsorption was 37% higher ($p < 0.05$) than under control conditions in the group acutely expanded (Table II) Prolonged saline infusion raised sodium reabsorption further by $36 \pm 18\%$ (Table III) parallel to $58 \pm 30\%$ increase in GFR

Binding of ouabain to microsomal Na-K ATPase from outer medulla

The average turnover number *i.e.* maximal catalytic rate per active site, for all animals where ouabain binding was measured (n = 21) equalled 5250 ± 340 min in the left kidney and 5430 ± 330 min in the right experimental kidney and there was no difference between the two kidneys in any experimental group (Fig. 5) Thus the increase in activity during infusion is caused by a higher concentration of enzyme.

From Table IV it is evident that the binding of ouabain to microsomal protein averaged 0.35 ± 0.01 nmol/mg protein (n = 42) Binding of ouabain was higher in the right than

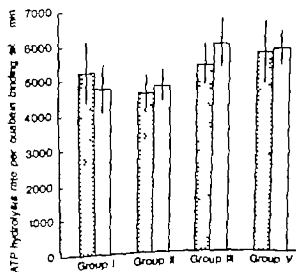


Fig. 5. Estimates of the maximal catalytic rate for the Na K ATPase from the (dotted columns: control) and right (columns experimental) kidneys in the experimental groups. Group I: In this group the left kidney was removed before the right. Group II Following ided nephrectomy hypertonic saline nfused for 20 min before the right kidney was also removed. Group III As group except that cycloheximide 5 mg kg was infused prior to removal of the kidney Group V Six days followed by hypertonic saline infusion for 2 h elapsed between the two nephrectomies.

TABLE IV Binding of ouabain to microsomes from rabbit renal outer medulla.

	Group I Control	Group II Acute expansion	Group III Cyclohex. + acute expansion	Group V Nephrect. + prolonged expansion
Right kidney (control)	0.37 ± 0.04	0.33 ± 0.03	0.32 ± 0.02	0.37 ± 0.02
Left kidney (experimental)	0.35 ± 0.05	0.37 ± 0.03	0.34 ± 0.02	0.45 ± 0.02
	NS	0.05	NS	
	(n = 5)	(n = 6)	(n = 7)	(n = 3)

Ouabain-binding in nmol ouabain/mg protein. Mean values ± standard error of the mean.

the left kidney only in the acutely infused group (Group II), but the microsomal Na-K ATPase activity was increased in parallel leaving the turnover number unchanged.

Jørgensen (1974) reports a higher turnover number probably due to the higher purity of preparation and different method of activation.

Discussion

The combined effects of extracellular volume expansion and a rise in plasma sodium concentration increase the Na-K-ATPase activity of outer medulla by 23 ± 4 %, whereas cortical activity is unchanged. Katz and Genant (1971) showed originally that the activity of rat renal enzyme increased by 12.5 % in response to i.v. isotonic saline infusion for 3 h, but in a later report, ascribed this to an artifact because the results were divergent when analysing a microsomal fraction (Katz and Lindheimer 1975). This study demonstrates that the functional significance of Na-K-ATPase activity is best expressed in relation to total kidney protein or mass, whereas the high specific activity obtained in the microsomal fraction only represents a variable fraction of total renal enzyme activity resulting in a poor correlation between activities in the crude homogenate and the microsomal fraction. Furthermore, changes in tissue enzyme concentration can not be detected if a highly purified preparation is analysed even if all the activity is recovered. The present experimental design allows paired comparison, and the extreme hypertonic extracellular expansion ensures a great increase in sodium reabsorption in the outer medulla (Kiil *et al.* 1971).

The enzymatic response to saline infusion developed within 20 min. Maximal catalytic rate per active site was not increased, as judged from the ratio of enzyme activity to bound ouabain in the microsomal fraction. Consequently the tissue concentration of enzyme was raised by either activation of latent enzymes or by rapid synthesis of new protein. The latter possibility is ruled out as the enzymatic response to saline infusion was unaffected following inhibition of protein synthesis at the ribosomal translation level with an effective dose of cycloheximide. The rapid adaptation of outer medullary Na-K-ATPase is thus due to an increase in the number of pumping sites by recruitment of inactive enzymes.

The mechanism of acute Na-K-ATPase activation might involve activation through protein kinase, association of the subunits, or some unspecific change of membrane configuration. However if the effect was demasking of intact enzymes, unaccessible to

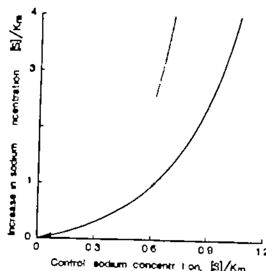


Fig. 6. Based on Michaelis-Menten kinetics it has been calculated what the rise in intracellular sodium concentration $\Delta S/K_m$ would have to be to obtain a doubling of the Na-K ATPase pumping rate starting at different concentrations S/K_m. The broken line is calculated assuming unchanged V_{max}, whereas the unbroken line takes into account a concomitant 23 % increase of V_{max}.

substrate, NaDOC treatment would be expected to abolish the difference between control and expanded kidneys. During homogenization, the plasma membrane forms vesicles which may be opened by NaDOC treatment, thereby increasing the *in vitro* enzyme activity. No similar protective mechanism of the Na-K ATPase exists *in vivo* as the difference in enzyme activity between the two kidneys was independent of the NaDOC effect.

It is not possible to quantify the change in sodium reabsorption in the diluting segment from the present data. Findings from micropuncture studies on rats (Landwehr *et al* 1967, Kunau *et al* 1974) support measurements in dogs of both free water clearance (Rector *et al* 1964) and renal metabolic rates (Kill *et al* 1971) showing that sodium reabsorption in outer medulla may be raised more than in other parts of the kidney during saline infusion. Katz and Lindheimer (1975) argue that an estimated increase of 100–200 % in sodium reabsorption in the outer medulla cannot be accounted for by the much smaller increase in Na-K-ATPase activity. However, as discussed in the following, an activation of latent enzymes amounting to 23 % in combination with an elevation of the intracellular sodium concentration may cause a great increase in the cellular pumping rate of sodium.

As proposed by Kill (1971), the luminal sodium concentration in outer medulla will be elevated during increased delivery of sodium to the diluting segment. Thus, intracellular sodium concentration will also be increased stimulating the Na-K ATPase in the peritubular membrane. A new steady state is reached where net flux of sodium through the cell is elevated and intracellular sodium concentration maintained at a higher level.

The *in vitro* activation of Na-K ATPase by sodium does not follow Michaelis-Menten kinetics because of allosteric interaction (Lindenmayer *et al* 1974), but the deviation is slight and the response to sodium may be described by an apparent K_m and V_{max} (Katz and Lindheimer 1975). If saline expansion did not change the number of pumping sites (V_{max}), intracellular sodium concentration should be well below K_m beforehand to obtain a doubling of the pumping rate. Even so, at a starting concentration of 0.61 K_m, the intracellular sodium concentration would have to rise by 2.5 K_m (Fig. 6, circle on broken line). However, taking into account the 23 % increase in enzyme activity (23 % increase in V_{max}), it is necessary to increase the intracellular concentration of so[illegible]nly 1 K_m (Fig. 6

de on unbroken line) to obtain the same doubling of the pumping rate. Hence, a small crease in the number of pumping sites amplifies the response to a rise in intracellular dium concentration. An implicit condition is that the enzymatic response to variations in dium concentration is identical before and after saline infusion, which has been demonstrated by others (Katz and Lindheimer 1975).

A consequence of this hypothesis is that effective intracellular sodium concentration must t be higher than K_m during hydropenic conditions if a doubling of the sodium reabsorption is to occur in response to increased delivery of sodium to the distal nephron. As the sponse of Na-K-ATPase to sodium is influenced by other cations such as Mg^{2+} Ca and (Skou 1960, Lindenmayer *et al.* 1974), no data are available on the *in vivo* K_m. Intracellular concentrations of sodium and potassium could vary inversely affecting enzyme tivity as shown in the right part of the curve in Fig. 2. However reliable estimates of tracellular ion concentrations in the renal outer medulla have not been made. As can be duced from Fig. 6, the response to an increment in intracellular sodium concentration greater the lower the starting concentration.

The total catalytic capacity in the whole kidney for ATP hydrolysis by Na-K-ATPase of the same order of magnitude as the sodium reabsorption during control conditions. Assuming that 2 3 sodium ions are transported per ATP hydrolyzed (Sejersted *et al.* 1976), e transport rate is 30–50 % of maximum providing renal Na-K-ATPase takes care of all odium reabsorption. This does not hold true, especially in the proximal tubules where the lk of reabsorbed sodium could pass through paracellular pathways (Mathisen *et al* 1976). the diluting segment, however where the Na-K-ATPase activity is highest (Schmidt and Dubach 1969), less than half of the filtered sodium load is reabsorbed and the enzymes therefore, must be working at less than 30% of their maximal catalytic rate.

The maximal enzymatic response to an increase in filtered load of sodium is 34 ± 10% higher specific activity of the outer medullary Na-K-ATPase following nephrectomy in combination with prolonged saline infusion. This is not significantly more than obtained by 0 min hypertonic saline infusion. Katz and Epstein (1967) reported a 25% increase in light microsomal Na-K-ATPase from whole rat kidneys 7 days postnephrectomy The present results show that this increase is confined to the outer medulla and that saline infusion, on top of the response to nephrectomy produces, if anything, only a very small further activation of latent enzymes. Thus, following unilateral nephrectomy the activation of renal Na K ATPase might not be due to the hypertrophy *per se* but might involve an activation of latent enzymes similar to the response obtained by acute increase in delivery of sodium to the diluting segment.

Because dry renal weight increased by 17 ± 4% following nephrectomy and the amount of renal protein even more, as evidenced by higher protein concentration (Malt 1969), how the Na-K-ATPase activity is expressed is of the utmost importance. Following nephrectomy and saline infusion, the total number of renal Na-K ATPase *molecules* is thus increased much more than 50%. Schmidt and Dubach (1974) observed very rapid elevation of cortical distal tubular Na-K-ATPase activity in relation to tubular dry weight which, compared to the unaltered cortical activity found in the present study must at least partly be ascribed to the smaller increment in tissue weight compared to the amount of total tissue protein.

Many factors, including dietary potassium or protein intake (Silva *et al* 1973, Field and Hayslett 1975 Katz and Epstein 1967), or administration of glucocorticoids (Ma *et al* 1968 Katz and Epstein 1967) seem to be important determinants for the lo regulation of renal Na K ATPase activity So far the only argument against a relation between sodium reabsorption in the diluting segment and Na K ATPase is that t activity remained unchanged when GFR was acutely or chronically reduced by clam of the renal artery (Nechay and Nelson 1970, Fisher *et al* 1975) In this situation, the red sodium reabsorption could be due to lower intracellular sodium concentration.

At least two mechanisms may account for acute alterations in sodium reabsorptio the diluting segment. Firstly alterations in intracellular sodium concentration may di affect the catalytic rate of renal Na K ATPase. Secondly latent enzyme is activated d saline infusion thereby increasing the concentration of active Na-K ATPase in the medulla.

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References

CAMERON, J M I *Fundament l formulas f physics* (Chapter 2 Statistics). Ed. D H Menzel I Publications, Inc, New York 1960 Vol. I 140

CHARNEY A N P SILVA and F H EPSTEIN An *in vitro* inhibitor of Na K ATPase present in an a sinetriphosphate preparation *J appl Physiol* 1975 *39* 156–158.

FINKELSTEIN F O nd J P HAYSLETT Role of med llary Na-K ATPase i renal potassium adapt *Amer J Physiol* 1975 *229* 524–528.

FISHER, K. A., L. G WELT and J P HAYSLETT Dissociation of Na K ATPase specific activity an reabsorption of sodium. *Amer J Physiol* 1975 *228* 1745–1749

FISKE, C. H a d Y SUBBAROW The colorimetric determination of phosphorus. *J biol. Chem.* 1925 375–400.

HEGYVARY C., Ouabain bind g nd phosphorylation of (Na + K)-ATPase treated with N-ethylmalei o oligomycin. *Biochim. biophys.* Acta (Amst.) 1976. *422* 365–379

HODGES, J L. and E. L. LEHMANN *Basic concepts of probabil ty and statistics*. Ed. E. L. Lehmann. Ho Day San Francisco. 1970 339–360

JØRGENSEN, P L., Purification and characterization f (Na + K)-ATPase. IV Estimation of the p and of the molecular weight and polypeptide content per enzyme un t i preparations from the medulla of rabbit kidney *Biochim. biophys. A ta* (Amst.) 1974 *356*. 53–67

JØRGENSEN, P L. and J C. S ou, Purification nd characterization of (Na + K)-ATPase. I The effe of detergents on the activity of (Na + K)-ATPase n preparations from the outer medulla of r kidney *Biochim. bioph) Acta* (Amst) 1971 *233* 366–380.

KATZ, A. I and F H EPSTEIN, The role f sodium potassium-activated adenosine triphosphatase i reabsorption of sodium by the kidney *J l n. Invest* 1967 *46* 1999–2011

KATZ, A I and H K. GENANT Effect of extracellular volume expa sion n renal cortical and medu N K ATPase. *Pflügers Arch. ges Physiol* 1971 *330* 136–148

KATZ, A. I and M D LINDHEIMER Relation of N K ATPase to cute changes i renal tubular so and potassium transport. *J gen Physiol* 1975 *66* 709–22.

KIIL, F N K ATPases as regulators of sodium nd water excretion. *Scand. J clin Lab. Invest* (edito 1971 *28* 375–378.

KIIL, F J JOHANNESEN and K. AUKLAND, Metabolic rat renal cortex and medulla during mann and saline infusion. *Amer J Physiol* 1971 *220* 565–570

[illegible], R. J. Jr., H. L. Wren and S. C. Borman, Characteristics of sodium reabsorption in the loop of Henle and distal tubule. *Amer. J. Physiol.* 1974. 227. 1181–1191.

[illegible], J. Immunoelectrophoretic determination of the distribution of (Na + K) ATPase over the plasma membranes of renal convoluted tubules. I. Distal segment. *J. Cell. Biol.* 1976. 68. 287–303.

[illegible], D. M., R. M. Klein and G. Giebisch, Renal tubular sodium and water reabsorption in the hypertonic sodium chloride-loaded rat. *Amer. J. Physiol.* 1967. 212. 1327–1333.

[illegible], M. O. M. Sejersted, M. Rayder and F. Kiil, Comparison of renal responses to ouabain and ethacrynic acid. *Amer. J. Physiol.* 1974. 226. 1221–1226.

[illegible], O. E., A. Schwartz and H. K. Thompson Jr., A kinetic description for sodium and potassium effects on (Na^{+}+K^{+})-adenosine triphosphatase: A model for two-nonequivalent site potassium activation and an analysis of multiequivalent site models for sodium activation. *J. Physiol.* (Lond.) 1974. 236. 1–28.

[illegible], O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* 1951. 193. 265–275.

[illegible], R. A. Compensatory growth of the kidney. *New Engl. J. Med.* 1969. 280. 1446–1459.

[illegible], A. K. Bensch and F. H. Epstein, (Na K)-activated ATPase in kidney cell membranes of normal and methylprednisolone-treated rats. *Biochim. biophys. Acta* (Amst.) 1968. 150. 563–571.

[illegible] Maldonado, M., G. Eknoyan, J. C. Allen, W. N. Suki and A. Schwartz, Urine dilution and concentration after digoxin infusion into the renal artery of dogs. *Proc. Soc. exp. Biol.* (N.Y.) 1970. 134. 855–860.

[illegible], O., M. Rayder, O. M. Sejersted and F. Kiil, Effect of acetazolamide on glomerulotubular balance and renal metabolic rate. *Scand. J. clin. Lab. Invest.* In press.

[illegible], B. R. and J. A. Nelson, Renal ouabain-sensitive adenosine triphosphatase activity and Na reabsorption. *J. Pharmacol. exp. Ther.* 1970. 175. 717–724.

[illegible], F. C. Jr., G. V. Gentry, F. Kiil and D. W. Seldin, Influence of expansion of extracellular volume on tubular reabsorption of sodium independent of changes in glomerular filtration rate and aldosterone activity. *J. clin. Invest.* 1964. 43. 341–348.

[illegible], U. and U. C. Dubach, Activity of (Na K)-stimulated adenosine-triphosphatase in the rat nephron. *Pflügers Arch. ges. Physiol.* 1969. 306. 219–226.

[illegible], U. and U. C. Dubach, Induction of Na K ATPase in the proximal and distal convolution of the rat nephron after uninephrectomy. *Pflügers Arch. ges. Physiol.* 1974. 346. 39–48.

[illegible], J. Microperfusion study of single short loops of Henle in rat kidney. *Pflügers Arch. ges. Physiol.* 1968. 300. 255–282.

[illegible], O. M., M. Lie and F. Kiil, Effect of ouabain on metabolic rate in renal cortex and medulla. *Amer. J. Physiol.* 1971. 220. 1488–1493.

[illegible], O. M., O. Mathisen and F. Kiil, Oxygen requirement of renal Na-K-ATPase-dependent sodium reabsorption. *Amer. J. Physiol.* In press.

[illegible], P. J. P. Hayslett and F. H. Epstein, The role of Na-K-activated adenosine triphosphatase in potassium adaptation. Stimulation of enzymatic activity by potassium loading. *J. clin. Invest.* 1973. 52. 2665–2671.

[illegible], J. C., Further investigations on Mg^{++} Na -activated adenosinetriphosphatase, possibly related to the active linked transport of Na^{+} and K across the nerve membrane. *Biochim. biophys. Acta* (Amst.) 1960. 42. 6–23.

[illegible], M. and M. Bensch, Electron microscopic study in several mammalian species of the reaction product enzymatically liberated from adenosine triphosphate in the kidney. *Lab. Invest.* 1964. 13. 476–489.

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Effect of Sodium Pentobarbital on the Apparent Turnover of Acetylcholine in Different Brain Regions

By

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Abstract

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The turnover rate of acetylcholine (ACh) was measured in six different brain regions in the mouse following pulse injection of radioactive choline (Ch) and killing of the animals by microwave irradiation of the head (0.25 s, 5 kW). The time course of the change in H ACh/³H-Ch ratio was linear 0-60 s in all brain regions after administration of H-Ch. Plots of the specific radioactivities (SA) of ACh and Ch versus time showed precursor-product relationship in all brain regions except the cerebellum both in control and pentobarbital anesthesized animals. The turnover was highest n the striatum (55 nmol g⁻¹ min⁻¹); in the cortex and hippocampus this value was app oximately half (27 and 21 nmol g⁻¹ min⁻¹); in midbrain and medulla oblongata the turnover rates were only 11 and 10 nmol g⁻¹ min⁻¹. Sodium pentobarbital anesthesia reduced specifically the turnover the cortex and hippocampus to about 60 per cent.

Key words: Turnover acetylcholine, choline, brain regions, sodium pentobarbital, microwave irradiation

Barbiturate anesthesia increases the steady state level of endogenous acetylcholine (Tobias *et al* 1946, Richter and Crossland 1949) and decreases its turnover in the brain (Schuberth *et al.* 1969) Studies of the effect of sodium pentobarbital on the steady state level of ACh in different brain regions have shown that barbiturates markedly increase the ACh level in the hippocampus and cerebral cortex, slightly in the midbrain but practically no change in the cerebellum, medulla oblongata and striatum (Nordberg and Sundwall 1973 1975). The biosynthesis of radioactive ACh is also most markedly reduced in the hippocampus and cortex. The turnover could not be estimated, however owing to a post mortem increase in endogenous choline (Ch).

The present paper describes experiments in which mice were killed by microwave irradiation, a technique which produces rapid enzyme inactivation and at the same time allows careful dissection of the brain (Stravinoha *et al* 1970 1974 Nordberg and Sundwall ... Both endogenous Ch and ACh as well as the rate of biosynthesis radioactive ACh

[illegible] measured in different brain regions. The results show that the ACh turnover differs markedly [illegible] ween different brain regions and is specifically decreased in the cerebral cortex and hippo[illegible]campus following treatment with sodium pentobarbital.

Materials and Methods

[illegible] mice (NMRI strain) weighing 20-24 g were supplied with food and water *ad libitum* and kept under [illegible] diurnal lighting (artificial) for at least 6 days. The light was switched on at 6 [illegible] and switched off [illegible] p. The animals were always killed between 10 [illegible] and 11.30 [illegible].

Sodium pentobarbital (60 mg/kg) in saline was injected intraperitoneally (i.p.) and 15 min later an i[illegible] [illegible] of 15 nmol of ^{3}H-Me-Ch (16.5 Ci/mmol) dissolved in 100 μl of saline was given in 2-3 [illegible] into [illegible] tail [illegible]. The rectal temperature was kept at 38°C by warming the animals with an infrared lamp. At different [illegible] points after the injection of ^{3}H-Me-Ch (0.5, 1, 5 or 10 min) the animals were killed by microwave irradiation of the head for 0.25 [illegible] (5 kW, 2450 MHz, Metabostat, Gerling Moore) (Nordberg and Sundwall [illegible]). Animals injected with saline instead of sodium pentobarbital served as controls.

The brains were rapidly removed, placed on an ice-cold glass plate and dissected into six discrete regions [illegible] (cerebellum, medulla oblongata + pons, midbrain (including hypothalamus), striatum, hippocampus and [illegible]) essentially according to the method described by Glowinski and Iversen (1966). The brain tissue [illegible] weighed, homogenized and extracted with 7 [illegible] ice-cold trichloroacetic acid (TCA). The homogenates [illegible] stored in [illegible] cold room ([illegible] 5°C) for 30 min and centrifuged at 4000 rpm for 10 min (for cortex 6000 [illegible], 15 min). The pellets were resuspended with TCA and centrifuged. To remove excess TCA the combined [illegible] were extracted with portions of ether until pH of 4.0 was reached. The extracts were freeze-[illegible] and dissolved in 100 μl glass-redistilled water. A sample (20 μl) of the extract was used for determination of the total radioactivity (^{3}*H-tot*) and the same volume was submitted to high voltage electrophoresis [illegible] separation of the principal metabolites [illegible]H-phosphorylcholine (PhCh), ^{3}H-ACh and ^{3}H-Ch. The buffer [illegible] 4.0) contained pyridine, acetic acid, acetone and water (8 8 30 154 [illegible]/v). After scanning the paper [illegible] in a Packard Radiochromatograph the radioactivity was measured by liquid scintillation. 10 μl [illegible] of the extract was used for determination of endogenous ACh using the dorsal muscle of the leech suspended [illegible] microbath (Nordberg and Sundwall 1976).

Endogenous Ch was estimated in separate homogenates. The brain regions were homogenized and [illegible] extracted with 1 M formic acid and acetone (15 85 [illegible]/v), kept in cold room for 30 min and centrifuged at [illegible]00 rpm for 15 min. The supernatants were extracted 3 times with heptane and chloroform (80 20 [illegible]/v) [illegible] the organic and lipid phases were carefully aspirated. Ch was separated by low voltage electrophoresis, [illegible] (pH 2.0 formic acid, glacial acetic acid and water (25 75 900 [illegible]/v)), eluted from the paper strips and labelled with ^{3}H-acetyl-CoA and choline acetyltransferase according to a modification (Nordberg and Sundwall 1976) of the method of Saelens *et al.* (1973).

Results

1. Effect of sodium pentobarbital on endogenous ACh and Ch in discrete brain regions

It is seen in Table I that sodium pentobarbital (60 mg/kg, i.p.) increased the endogenous steady state level of ACh by about 90% in the hippocampus, 70% in the cortex and about 20% in the midbrain. No effect was noted in the striatum, medulla oblongata or cerebellum.

In contrast, endogenous Ch was fairly evenly distributed and no significant effect was observed with the exception of an increase in the cerebellum ($p < 0.05$).

2. Effect on biotransformation of [illegible]*H-Ch in discrete brain regions*

The results are summarized in Fig. 1-4. As seen in Fig. 1, sodium pentobarbital decreased ($p < 0.001$) the initial rate of biosynthesis of ^{3}H-ACh in the hippocampus (by about 75%), cortex (about 65 [illegible]) and midbrain (about [illegible]

TABLE I Effect of sodium pentobarbital (60 mg/kg i.p.) on the steady state levels of endogenous ch[illegible] and acetylcholine (nmol g⁻¹) in discrete mouse brain regions. The animals were killed by m[illegible] wave irradiation of the head (0 25 s 5 kW) 16 min after administration of sodium pentobarb[illegible]

Brain region	Choline		Acetylcholine	
	Control	Sodium pentobarbital	Control	Sodium pentobarbital
Cerebellum	32.8±4 63 (10)	47.3±3 49 (8)	5.2±1 05 (4)	3 7±0.73 (4
Medulla oblongata	43.8±4.30 (8)	37.2±4 89 (4)	29 1±1 10 (4)	32 5± [illegible]
Midbrain	33 8±3.56 (9)	44 7±7.80 (7)	29.0±1.80 (6)	35 5±1 27 (4
Striatum	38. ±3 11 (10)	45 9±6.30 (9)	76.8±1 78 (5)	81.3± [illegible]
Hippocampus	[illegible]±3 18 (8)	32.2±3 69 (8)	21.2±[illegible]32 (6)	39 8±0.23 (5
Cortex	33.8±3 34 (11)	39 0±2.26 (9)	24 8±1 44 (6)	41.1±1.27 (4

Mean value (M)±S.E. (n) = number of expts. $p < 0.001$ $p < 0.05$.

in the cerebellum, *medulla oblongata* and striatum. The H-Ch concentration was [illegible] 25% higher in the cortex and hippocampus than in the controls (Fig. ?).

The rate of H PhCh biosynthesis was increased at 1 min in the cortex and hippocamp[illegible] (Fig. 3)

The time course of the change in the ³H ACh/³H-Ch ratio was linear at 0–60 s in all bra[illegible]

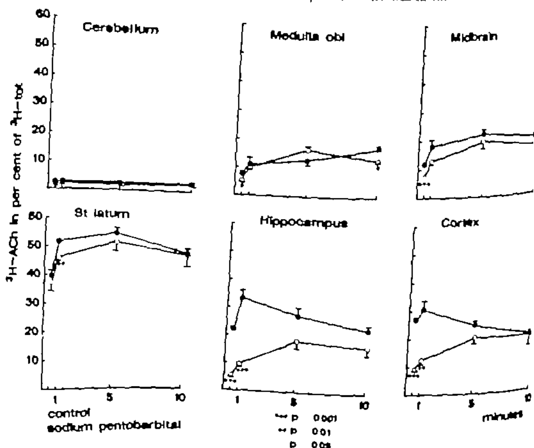


Fig. 1 Effect of sodium pentobarbital (60 mg/kg i.p) on the biosynthesis of H-ACh in different brai[illegible] regions. Each point represents the mean value of 3–6 expts. Vertical bars [illegible]

Fig. 2. Effect of sodium pentobarbital (60 mg/kg i.p.) on the concentration of untransformed ^{3}H-Ch in different brain regions. Each point represents the mean value of 3–6 expts. Vertical bars indicate S.E.

Fig. 3. Effect of sodium pentobarbital (60 mg/kg i.p.) on the biosynthesis of ^{3}H-PhCh in different brain regions. Each point represents the mean value of 3–6 expts. Vertical bars indicate S.E.

TABLE I Effect of sodium pentobarbital (60 mg/kg i.p.) on the steady state levels of endogenous choline and acetylcholine (nmol g⁻) in discrete mouse brain regions. The animals were killed by microwave irradiation of the head (0.25 s 5 kW) 16 min after administration of sodium pentobarbital

Brain region	Choline		Acetylcholine	
	Control	Sodium pentobarbital	Control	Sodium pentobarbital
Cerebellum	32.8 ± 4 63 (10)	47.3 ± 3 49 (8)*	5 2 ± 1.05 (4)	5 7 ± 0 73 (4)
Medulla oblongata	43 8 ± 4.30 (8)	37.2 ± 4.89 (4)	29 1 ± 1 10 (4)	32 5 ± 2.38 (4)
Midbrain	33 8 ± 3.56 (9)	44 7 ± 7 80 (7)	29 0 ± 1.80 (6)	35.5 + 1 27 (4)*
Striatum	38.2 ± 3 11 (10)	45.9 ± 6.30 (9)	76.8 ± 1 78 (5)	81 3 + 2 12 (4)
Hippocampus	28.2 ± 3.18 (8)	32.2 ± 3 69 (8)	21.2 ± 2.32 (6)	39 8 + 0 23 (3)**
Cortex	33.8 ± 3 34 (11)	39 0 ± 2.26 (9)	24 8 ± 1 44 (6)	43.1 + 1 77 (4)**

Mean value (Mv) ± S.E. (n) = number of expts. p < 0.001 p < 0.05

in the cerebellum, medulla oblongata and striatum. The H-Ch concentration was about 25 % higher in the cortex and hippocampus than in the controls (Fig. 2).

The rate of H PhCh biosynthesis was increased at 1 min in the cortex and hippocampus (Fig. 3)

The time course of the change in the H ACh/³H-Ch ratio was linear at 0–60 s in all brain

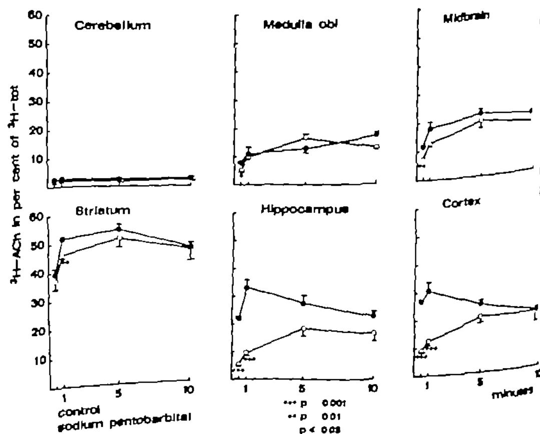


Fig. 1 Effect of sodium pentobarbital (60 mg/kg i.p.) on the biosynthesis of H-ACh in different brain regions. Each point represents the mean value of 3–6 expts. Vertical bars indicate S.E.

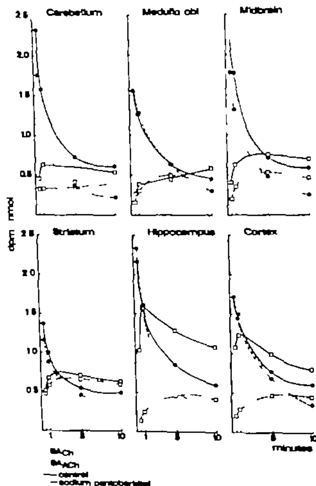


Fig. 3. Effect of sodium pentobarbital (60 mg/kg i.p.) on the specific radioactivities (SA) of ACh and Ch in different brain regions. SA are expressed as ^{3}H-ACh and ^{3}H-CH in per cent of ^{3}H-ACh nmol endogenous ACh and Ch.

time taken to obtain complete inactivation of certain brain enzymes. In a previous paper Nordberg and Sundwall (1976) reported progressively higher values when three different methods of sacrifice were compared, viz. dislocation of the spine, whole body microwave irradiation (1.3 kW 7) and head irradiation (5 kW 0.25 s). The ACh in the striatum and cerebral cortex, in particular appears to be extremely sensitive. Radioactive ACh synthesized by the brain from radioactive Ch appears to be more easily preserved than endogenous ACh, since maximum levels are obtained with both the whole body and head irradiation procedures, whereas dislocation of the spine gives low values, especially in the striatum (Nordberg and Sundwall 1976).

Following anaesthesia with sodium pentobarbital (60 mg/kg. i.p.), dislocation of the spine yields markedly increased steady state levels of ACh in the cortex, hippocampus and midbrain (130 90 and 40%) but practically unchanged levels in the cerebellum, medulla oblongata and striatum (Nordberg and Sundwall 1975). The relative effects of sodium pentobarbital (per cent increase relative to control) are thus the same as when the animals are

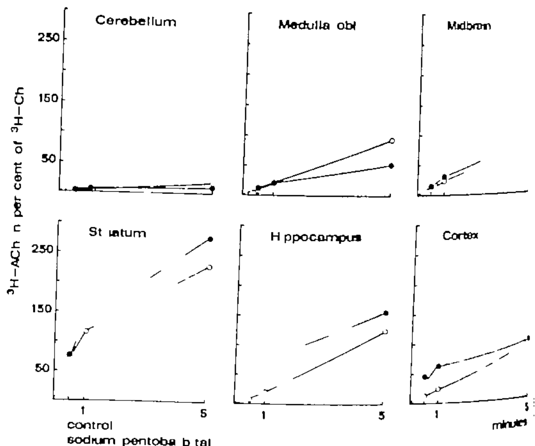


Fig. 4 Effect of sodium pentobarb tal (60 mg/kg i.p.) on the H ACh/^{3}H-Ch ratio in different brain re gions. Each poi t represents the mean value of 3–6 expts.

regions and was markedly decreased (by 60–80%) by sodium pentobarbital in the hippo campus and cerebral cortex (Fig. 4)

Effect on ACh turnover

The time courses of the changes in the specific radioactivities of ACh and Ch in the different brain regions are shown in Fig. 5 and Table II gives the apparent turnover calculated from the equation

$$\frac{\text{H–ACh}\quad \text{Ch nmoles}}{\text{H–Ch}\quad \text{g}\quad \text{min}} = \text{ACh nmol g}^{-1}\ \text{min}^{-1}$$

As seen in the table, only in the cortex and hippocampus was the turnover markedly reduced after treatment with sodium pentobarbital.

Discussion

Microwave irradiation is used increasingly for killing small rodents when the steady state level of labile compounds such as ACh are to be studied Generally higher values are found when this technique is used and it is obvious that the values obtained are related to the

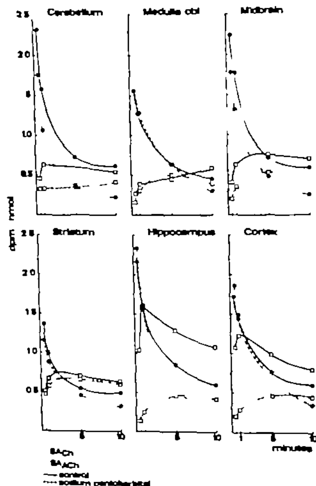


Fig. 5. Effect of sodium pentobarbital (60 mg/kg i.p.) on the specific radioactivities (SA) of ACh and Ch in different brain regions. SA are expressed as ^{3}H-ACh and ^{3}H-Ch in per cent of ... nmol endogenous ACh and Ch

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TABLE II Effect of sodium pentobarbital on the apparent turnover rate (nmol g^{-1} min^{-1}) of acetylcholine in different brain regions.

Brain region	Control	Sodium pentobarbital
Cerebellum	(2)	(2)
Medulla oblongata	10	7
Midbrain	11	9
Striatum	55	59
Hippocampus	21	6
Cortex	27	10

killed by irradiation of the head (this paper) A different relative effect is obtained after whole body irradiation with the 1.3 kW irradiation source In addition to the increase in ACh in the cortex, the hippocampus and midbrain, a marked increase is also found in the striatum and medulla oblongata (Nordberg and Sundwall 1975 unpublished data). The absolute ACh levels in nmol g⁻ after administration of sodium pentobarbital, on the other hand, are exactly the same following the two types of microwave irradiation. It is the control values that differ A possible explanation for these findings is that sodium pentobarbital stabilizes a very labile pool of endogenous ACh and/or that the anesthesia prevents or diminishes a heavy burst of ACh release to the synaptic cleft induced by the killing. This could explain the low values generally found after dislocation of the spine. This burst of ACh released into the synaptic cleft may be preserved by the rapid enzyme inactivation or it could be prohibited by the rapid tissue fixation produced by the microwave irradiation The different results obtained with regard to endogenous and newly synthesized radioactive ACh may have a bearing on this, indicating that at least two pools of ACh are involved. In a recent paper Atweh and Kuhar (1976) found endogenous ACh in the hippocampus more labile to destruction than H ACh

Also regarding the effect of sodium pentobarbital on endogenous ACh in the striatum, results differing from ours have been published Modak *et al* (1976) found an increase from 81 to 117 nmol g (44%) in the striatum of mice killed by irradiation of the head for 0 300 s (6 kW) and Trabucchi *et al.* (1975) found an increase from 54 to 89 nmol g^{-1} (65%) in the striatum of rats following microwave irradiation of the head for 1.2 s (2.5 kW). The reason for these discrepancies is unknown.

For estimation of the ACh turnover an intravenous pulse injection of tracer amounts of radioactive Ch has been used (Schuberth *et al* 1969). In the present study it has been ascertained that the ratio of radioactive ACh to Ch increases linearly with time in all brain regions, both in normal and sodium pentobarbital treated mice over the time interval 0 to 1.0 min (head irradiation) A precursor product relationship is also indicated by the fact that plots of the time course of the specific radioactivities (SA) of ACh and Ch intersect at the maximum SA of the product (ACh) in all brain regions except the cerebellum. This means that a basic criterion for a precursor product relationship would be fulfilled if in addition it were plausible that we have steady state instant mixing and an open single compartment (Zilversmit *et al* 1943). If this is assumed, it is possible to make a rough estimate of the turnover in the different regions. The highest turnover rate is found in the striatum (55 nmol

-¹). In the hippocampus and cortex the turnover is about half this value and in lla oblongata and midbrain about one fifth. Sodium pentobarbital has a very speci- on the turnover in the cortex and hippocampus. No other regions are affected. A elative effect has been found in the cortex and striatum by Trabucchi *et al.* (1975). ive effect of sodium pentobarbital anesthesia on the sodium dependent high affinity ptake in synaptosomes from different brain regions have been demonstrated by *et al.* (1976). The anesthesia decreased uptake in the hippocampus and cortex but effect on striatal synaptosomes. Regarding calculation of turnover rate, available are rather imperfect and different values have been published. The figures given by hi *et al.* (1975 table 2) for the striatum (18 nmol g min) and cortex (3 nmol g^{-1} are considerably lower than ours. They used a 6 min i.v. infusion of phosphoryl-. In this case the difference may be due to the longer time interval (0–6 min) used for on of the rate of conversion of radioactive Ch into radioactive ACh. The time course adioactive ACh/Ch ratio, obtained upon pulse injection is biphasic and the slope is rably less steep between 1 and 5 min. If this is also true following infusion of phos-choline an explanation for the low turnover figures is found. At 5 min our values are l nmol g min for the cortex and stratum, respectively. Owing to the rapid turn- ACh and the possibly preferential release of recently synthesized ACh with efficient of the products of hydrolysis, the longer time points tend to give low values.

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References

, S. F. and M. J. K. ..., Effects of anesthetics and septal lesions and stimulation on ³H-acetyl-ine formation in rat hippocampus. *Europ. J. Pharmacol.* 1976, 37: 311–319.

..., J. and L. L. Iversen, Regional studies of catecholamines in the rat brain.—I. *J. Neurochem.* 13: 655–669.

..., A. T. S. T. Weintraub, T. H. McCoy and W. B. St... Use of 300-msec irradiation for ... inactivation: A study of effects of sodium pentobarbital on acetylcholine concentration in mouse ... regions. *J. Pharmacol. exp. Ther.* 1976, 197: 245–252.

..., A. and A. Sundwall, Effect of pentobarbital on the turnover of acetylcholine in discrete ... of mouse brain in vivo. *Acta physiol. scand.* 1973, Suppl. 396, 69 (abstract).

..., A. and A. Sundwall, Effect of pentobarbital on endogenous acetylcholine and biotrans-... of radioactive choline in different brain regions. In: *Cholinergic Mechanisms*. Ed. P. G. Waser ... Press. New York. 1975. pp. 229–239.

..., A. and A. Sundwall, Biosynthesis of acetylcholine in different brain regions in vivo following ... methods of sacrifice by microwave irradiation. *Acta physiol. scand.* 1976, 98: 307–317.

..., D. and J. Crossland, Variation in acetylcholine content of the brain ... physiological state. ... *J. Physiol.* 1949, 159: 247–255.

..., I. K., I. P. Spiker, M. P. Allen and C. A. Cosnoy. Measurement of choline (Ch), acetylcholine (ACh), and their metabolism by combined enzymatic and radiometric techniques. In: *Methods of Neuro-chemistry* Vol. 4. Ed. R. Fried. Marcel Dekker Inc. New York. 1973. pp. 69–95.

..., J. B. Spare and A. Sundwall, A technique for the study of acetylcholine turnover in mouse ... in vivo. *J. Neurochem.* 1969, 16: 695–700.

..., J. R., S. Atweh and M. J. Kuhar, Sodium-dependent high affinity choline uptake: A regulatory ... in the synthesis of acetylcholine. *J. Neurochem.* 1976, 26: 909–912.

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Acta physiol. scand. 1977 99 345–352

From the Department of Urology, University Hospital, Lund, the Institute of Anatomy and Histology, University of Lund, Sweden, and the Division of Clinical Pharmacology, Institute of Pharmacology, University of Århus, Denmark

Adrenergic and Cholinergic Nerves of the Human Urethra and Urinary Bladder. A Histochemical Study

By

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Abstract

Ek, A., P. Alm, K.-E. Andersson and C. G. A. Persson. *Adrenergic and cholinergic nerves of the human urethra and urinary bladder. A histochemical study.* Acta physiol. scand. 1977. 99. 345–352.

The occurrence and distribution of adrenergic and acetylcholine esterase (AChE) positive nerves in the human urethra and urinary bladder were studied histochemically with the fluorescence method of Falck and Hillarp, and the copper thiocholine method of Koelle and Friedenwald. Both types of nerves were mainly confined to the layers of smooth muscle cells in the walls of the organs. In all parts of the urethra, there was a scanty supply of adrenergic nerves. Few adrenergic nerves were also found in the urinary bladder except in the trigone area, where they were abundant. AChE-positive nerves were uniformly and richly distributed in the urinary bladder. Throughout the urethra the distribution of AChE-positive nerve fibres was uniform, but the number was clearly less than in the urinary bladder. No intramurally located adrenergic or AChE positive ganglion cells could be demonstrated.

The function of the parasympathetic nerves of the urinary bladder is well known from physiological and pharmacological investigations in man as well as in other species. The occurrence and distribution of acetylcholine esterase (AChE) positive nerves (supposed to be cholinergic) in the detrusor muscle have been described in various animals, but, surprisingly only very briefly and incompletely in man. Studies in normal man and in patients with various types of bladder dysfunction, performed *in vitro* and *in vivo* (Todd and Mack 1969, Nergårdh and Boreus 1972, Awad *et al.* 1974, Nergårdh and Gierup 1974, Calne *et al.* 1975, Ek *et al.* 1976, Sundin *et al.* 1976), have indicated an adrenergic influence on the bladder and bladder neck. Adrenergic nerve terminals, supposed to be the anatomical background for these effects, have been demonstrated in various bladder parts of different animals, but, so far only in the detrusor muscle and trigone area of man (Mobley *et al.* 1966, Sundin *et al.* 1976).

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STAVINOHA, W B B PEPELKO and P W SMITH, Microwave radiation to inactivate cholinesterase in rat brain prior to analysis for acetylcholine. *Pharmacologist* (Wash.) 1970. *12* 257 (abstract)

STAVINOHA, W B S. T WEINTRAUB and A T MODAK, Regional concentration of choline and acetylcholine in the rat brain *J Neurochem.* 1974 *23* 885–886.

TOBIAS, J M M A LIPTON and A. A. LEPINAT Effect of anesthetics and convulsants on brain acetylcholine content. *Proc Soc exp Biol.* (N Y). 1946. *61* 51 54

TRABUCCHI, M D L. CHENEY G RACAGNI and E. COSTA, Pentobarbital and in vivo turnover rate of acetylcholine in mouse brain and in regions of rat brain *Pharmacol Res. Commun* 1975. *7* 81 94

ZILVERSMIT D B C. ENTENMANN and M C. FISCHLER, On the calculation of "turnover time" and "turnover rate" from experiments involving the use of labeling agents. *J gen Physiol.* 1943 *46*. 325-331

thiocholine method of Koelle and Friedenwald (1949), as modified by Holmstedt (1957), was used. Cryostat sections (about 10 μm) were incubated for 10 h in acetylthiocholine iodide after preceding treatment with mipafox (bis-monoisopropylamide phosphoryl fluoride, Swedish National Defence Research Inst.) for the inhibition of non-specific cholinesterase (Holmstedt 1957, Holmstedt and Sjöqvist 1961) and/or BW 284 C 51 (1,5-bis 4-allyldimethyl ammoniumphenyl pentane-3-one-dibromide, Wellcome Res. Lab., England) for the inhibition of specific AChE-activity (Bayliss and Todrick 1956). After counterstain in eosin, mounting was performed in Entellan (Merck).

Results

Adrenergic nerves

A considerable autofluorescence appeared in sections from all tissue specimens examined. It was mainly confined to the connective tissue in which bright yellowish fluorescent smooth filaments appeared. These filaments had a uniform thickness along their whole extensions and ran with wave-like course more or less irregularly and dense to each other. The autofluorescence did not seemingly change qualitatively or quantitatively whether or not omitting the formaldehyde treatment step of the Falck and Hillarp technique for the very induction of the specific adrenergic nerve fluorescence. On the other hand, for differentiation, the adrenergic nerve terminals always demonstrated a distinct beaded appearance displaying a somewhat more greenish fluorescence. They were never seen when the formaldehyde treatment step was excluded. No evident difference in the number and distribution of fluorescent adrenergic nerves could be seen between irradiated and non-irradiated tissue specimens. The bundles of smooth muscle cells, forming the most basally located urethral structures, appeared distinctly dark against the autofluorescent connective tissue.

In both the male and female urethras, there was generally a very scarce supply of adrenergic nerves (see Fig. 2 a and b). Around the slender bundles of smooth muscle cells, very fine varicose adrenergic nerve terminals in a small number were seen. Mostly they ran longitudinally along the bundles of smooth muscle cells, but sometimes transversely running terminals could also be observed, connecting the longitudinally running terminals to a more or less distinct plexus of terminals around the bundles of smooth muscle cells. Adrenergic terminals were also found around some blood vessels, mostly occurring in the smooth muscle layers. The number and distribution of adrenergic nerve terminals was appreciably the same in male and female urethras and did not differ between the various urethral parts.

The urinary bladder also displayed a very scarce supply of adrenergic nerves. Except in the trigone area, terminals appeared in a very restricted number around bundles of smooth muscle cells in the lateral walls of the bladder in the same way as in the urethra (*cf* Fig. 2 a and b). Some blood vessels supplied with adrenergic terminals were also seen. In the trigone area, the number of adrenergic terminals around bundles of smooth muscle cells was much greater (see Fig. 2 c). The fluorescence picture was the same in male and female urinary bladders. No adrenergic ganglion cells could be seen, neither in the urinary bladder nor in the urethra.

AChE-positive nerves

In the urinary bladder of both males and females, the number of AChE-positive nerves was generally much greater than that of adrenergic nerves. Around the bundles of smooth muscle cells of the lateral walls of the bladder slender AChE-positive fibres occurring in a similar

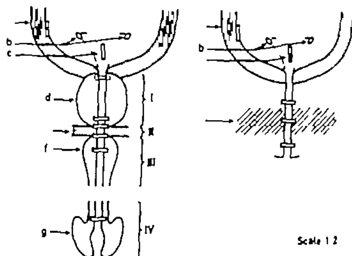


Fig. 1 Schematic picture of the male (left figure) and female (right figure) urethra and urinary bladd .ome adjacent structures. Site of location for the examined pieces of bladder (□) and urethra (□) static urethra, *II* Membraneous urethra, *III* Bulbous urethra, *IV* Penile urethra. *a*. Urinary bladde *b* Orifices for ureters, Trigone area, *d*. Prostate *e* Urogenital diaphragm, *f* Corpus cavernosum ur *g* Glans penis.

During the last few years, several reports have stressed the clinical importance o adrenergic influence on the urethra. The therapeutic results of decreasing urethral resis by α-adrenoceptor blocking agents in patients unable to empty their bladder for va reasons (Abel *et al* 1974 Khanna and Gonick 1975 Stockamp and Schreiter 1975 McG *et al* 1976, Whitfield *et al* 1976) seem to be well documented In patients suffering urinary stress incontinence therapeutic effects of α adrenoceptor stimulating agents (Di and Taub 1975 Schreiter *et al* 1976, Stewart *et al* 1976) and β-adrenoceptor blocking (Gleason *et al* 1974) have also been reported

Because of the scanty information on the innervation of the human urethra, and in c to better understand urethral mechanisms in further studies on urinary incontinence voiding disturbances in man, the present investigation was performed. It describe occurrence and distribution of adrenergic and AChE-positive nerves in the human ure and the detrusor muscle and trigone area of the urinary bladder

Material and Methods

In 5 men and 1 woman, who had received 4 500 rad preoperatively nd 3 men nd 2 omen, not operatively irradiated, total urethrocystectomy was performed because of locally wide spread an infiltrating carcinoma of the bladder Droperidol (Dridol Leo, Sweden), fentanyl (Leptanal® Leo, Swe and pancuron (Pavulon Organon, Holland) were given for anesthesia and premedic medica Quickly after removal of the bladder tissue specimens were taken from macroscopically tumor free a of the lateral walls and trigone area of the urinary bladder and from different parts of the urethra Fig. 1). The specimens were then rapidly frozen i nopentane cooled by liquid nitrogen. For the fluo cence histochemical demonstration of adrenergic nerves according to the technique of Falck and Hil pieces of tissue were freeze-dried, exposed to gaseous formaldehyde of standardized humidity and bedded in paraffin. Sections for fluorescence microscopy could then be made (for details, see Björkl *t al*. 1972). For the histochemical demonstration of acetylcholine-esterase (AChE) activity the sec

thiocholine method of Koelle and Friedenwald (1949), as modified by Holmstedt (1957), was used. Cryostat sections (about 10 μm) were incubated for 10 h in acetylthiocholine iodide after preceding treatment with inhibitor (bis-monoisopropylamide phosphoryl fluoride, Swedish National Defence Research Inst.) for the inhibition of non-specific cholinesterase (Holmstedt 1957, Holmstedt and Sjöqvist 1961) and/or BW 284 C 51 (1-5-bis 4-allyldimethyl ammoniumphenyl pentane-3-one-dibromide, Wellcome Res. Lab., England) for the inhibition of specific AChE-activity (Bayliss and Todrick 1956). After counterstain in [illegible], mounting was performed in Entellan (Merck).

Results

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Fig. 2 a. Urethra. The bundles of smooth muscle cells are transversely sectioned and seen i the figure dark areas surrounded by close encircli g adrenergic nerve terminals (↖). Fluorescence micrograph, 175

Fig. 2 b Urethra Longitudinal section of a bundle of smooth muscle cells seen i the figure as a dark area surrounded by bright utofluorescence from fibrous tissue Fine varicose adrenergic nerve terminals (↖) are seen in the bundle of smooth muscle cells along its longitudinal direction Fluorescence micrograph, 175

Fig. 2 c. Urinary bladder trigone area N merous adrenergic nerve terminals with high fluorescence intensity and a somewhat irregular course occu see .g (↖). Fluorescence micrograph, 175

number and with the same distribution in all regions of the bladder formed net-like plexu, see Fig. 3 a. The muscle bundles had a more dense supply of AChE positive nerves than of fluorescent adrenergic nerve terminals. In the basal layers of smooth muscle cells, large AChE positive nerve trunks appeared. In some of the blood vessels of these layers, AChE positive nerve fibres were found to end in the vascular adventitia

Both the male and female urethra demonstrated a similar pattern of distribution of AChE-activity as here described for the urinary bladder However in the urethra the occurrence of AChE positive nerve fibres around the bundles of smooth muscle cells was not as dense as in the bladder It seemed to be more dense than the corresponding occurrence of fluorescent adrenergic nerve terminals, see Fig. 3 b The distribution and number of AChE positive nerve fibres were not found to differ between various parts of the urethra No AChE positive ganglion cells were observed in the lateral walls or in the trigone area of the urinary bladder or in the urethra. The number and distribution of AChE-positive nerves were similar in irradiated and non-irradiated tissue specimens.

Discussion

For the histochemical demonstration of adrenergic nerves, the fluorescence method of Falck and Hillarp is widely used. Its very prominent sensitivity and specificity is well known and



Fig 3 Urinary bladder Rich distribution of AChE-positive nerves in the somewhat dark counterstained areas representing bundles of smooth muscle cells. Light micrograph, 110

Fig 3 b. Urethra. Compared with Fig 3 there is less AChE-positive nerves in relation to the bundles of smooth muscle cells Light micrograph, 110

has been thoroughly discussed (Björklund *et al* 1972). To demonstrate AChE-positive nerves, the copper thiocholine technique of Koelle and Friedenwald is at present one of the most widely used methods. However it is less sensitive and specific than the Falck and Hillarp technique for adrenergic nerves. To get the optimum and most specific demonstration of AChE-positive nervous structures, various methodological conditions should be fulfilled and always tested for each species and tissue studied (*cf* Eränkö 1966). This was done in pilot experiments in this study

The present investigation showed that there was a very scarce supply of adrenergic nerve terminals in the lateral part of the bladder compared to the rich supply in the trigone area. On the other hand, there was a rich supply of AChE-positive fibres, which was appreciably the same in the investigated bladder regions. This was valid both in parts displaying very few adrenergic nerves and in the trigone area. In view of this, it seems reasonable to assume that the copper thiocholine method almost exclusively demonstrates AChE-positive nerves and not, or to a very small extent, adrenergic terminals.

The number and general distribution of adrenergic nerves in the human urinary bladder reported here and recently by Sundin *et al* (1976), seem to agree with findings in other species such as cat, dog, rabbit, rat, and guinea-pig (Hamberger and Norberg 1965 El-Badawi and Schenk 1966, Owman and Sjöberg 1972, Wakade and Kirpekar 1972, Raezer *et al.* 1973 1976, Sundin and Dahlström 1973, Dixon and Gosling 1974 Alm and Elmér 1975) Also the uniform, rich supply of AChE-positive fibres in the human urinary bladder only very briefly and incompletely described before (Mobley *et al* 1966, Nyo 1969 Faerman

et al 1971) is similar to findings described in cat, dog, rabbit, rat, and guinea-pig (El-Badawi and Schenk 1966 Raezer *et al.* 1973 1976, Dixon and Gosling 1974 Alm and Elmér 1975) However the present work demonstrates a very sparse supply of adrenergic nerves in all parts of the human urethra. This is in contrast to observations in other species such as cat, dog, guinea-pig, and rat, demonstrating a rich supply of adrenergic nerve terminals, somewhat denser in the proximal part of the organ (Owman *et al* 1971 Owman and Sjöberg 1972, Sundin and Dahlström 1973 Benson *et al* 1976, Sjöberg *et al.* 1976). Furthermore the present study describes a rather rich supply of AChE positive nerves in all parts of the human urethra. This is interesting, as there seem to be no earlier studies in any species describing AChE-positive nerves in the urethra. Thus, the occurrence and distribution of adrenergic and AChE positive nerves of the human urinary bladder are in accordance with findings in several other species. On the other hand, the human urethra seems to differ in these respects from other species.

The uniform although scarce occurrence of adrenergic nerves throughout the human urethra suggests a constant relationship between nerve terminals and muscle cells. This finding also suggests that there are no functional differences between the effects exerted by the adrenergic nerves in the different parts of the organ. This view is supported by studies showing a uniform contractile response of the smooth muscle in all parts of the human urethra induced by α-adrenoceptor stimulating drugs (Ek *et al* 1976)

The functional importance of the AChE positive nerves of the urethra is unknown. Studies *in vitro* on the isolated human urethra showed that exogenous acetylcholine, even when administered in high concentrations, had insignificant effects (Ek *et al.* 1976). This seems somewhat contradictory to the occurrence of AChE positive nerves forming net-like plexa around the bundles of smooth muscle cells. However one explanation might be that cholinergic nerves of the human urethra do not directly exert physiological influence on the smooth muscle cells. Hypothetically they could form synapses with the adrenergic nerves and in some way interact with these in the contractile response. Interaction between adrenergic and cholinergic nerves was originally suggested by Burn and Rand (1959 1965) and has later been extensively studied (see Sporrong 1970)

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References

ABEL, B J N. O K. GIBBON, R. M J [illegible]SON and K. R. KRISHNAN The neuropathic urethra. *Lancet* 197[illegible] *II* 1229–1230.

ALM, P and M ELMÉR, Adrenergic nd cholinergic innervation of the rat urinary bladder *Acta physiol scand.* 1975 94 36–45

AWAD S. A., G B UCE, J W CARROCAMPI, M LIN and G S MARKS, Distribution of α- and β-adrenoceptors in human urinary bladder *Brit J Pharmacol* 1974 50, 525–529

BAYLISS, B. J and A. TODRICK The use of selective acetylcholinesterase inhibitor in the estimation of pseudocholinesterase activity in rat brain. *Biochem. J* 1956. 62 62–67

BENSON G S D JACOBOWITZ, D M RAEZER, J N CORRIERE, JR. and A. J WEIN, Adrenergic innervation and stimulation of canine urethra. *Urology* 1976. 7 337 340.

BJÖRKLUND A., B FALCK and CH. OWMAN, Fluorescence microscopic and microspectrofluorometric tech iques for the cellula localization nd characterization of biogenic amines. In. S. A. Ber[illegible]

Methods of investigation and diagnostic endocrinology 1 (J. E. Rall and I. J. Kopin, eds.), The thyroid and biogenic amines, p. 318–368. Amsterdam: North Holland Publ. Comp. 1972.
…, J. H. and M. J. Rand, Sympathetic postganglionic mechanism. *Nature* (Lond.) 1959 *184* 163–165.
…, J. H. and M. J. Rand, Acetylcholine in adrenergic transmission. *Ann. Rev. Pharmacol.* 1965. *5*. 163–182.
…, M. S. Raz and M. Zeigler, Adrenergic and cholinergic receptors in the human prostate, prostatic capsule and bladder neck. *Brit. J. Urol.* 1975. *47* 193–202.
…, A. C. and M. Taub, Ephedrine in the treatment of urinary incontinence. *Urology* 1975 *5* 624–626.
…, J. R. and J. A. Gosling, The distribution of noradrenergic nerves and small, intensely fluorescent (SIF) cells in the cat urinary bladder. *Cell Tiss. Res.* 1974. *150*. 147 159
…, B. Cholinesterase in ocular and orbital tissues of some mammals. *Acta Univ. Lund.* 1966. *II* No 7
…, A. P. Alm, K.-E. Andersson and C. G. A. Persson, Adrenoceptor and cholinoceptor mediated responses of the isolated human urethra. *Scand. J. Urol. Nephrol.* In press.
…, A. and E. A. Schenk, Dual innervation of the mammalian urinary bladder. *Amer. J. Anat.* 1966 *119* 405–420
…, I. L. Flocks, D. Gelfand, M. Jadeepnsky, D. Fox, M. Malik and E. Alvarez, Autonomic nervous system and diabetes: Histochemical study of the autonomic nerve fibres of the urinary bladder in diabetic patients. *Diabetes* 1971 *20*, Suppl. 1 331 332.
…, D. M., R. J. Krane, M. R. Bottaccini and M. Pierce, The urethral continence zone and its relation to stress incontinence. *J. Urol.* (Baltimore) 1974. *112*. 81–88.
…, B. and K.-A. Norberg, Adrenergic synaptic terminals and nerve cells in bladder ganglia of the cat. *Int. J. Neuropharmacol.* 1965. *4* 41–45.
…, B. A modification of the thiocholine method for determination of cholinesterase. II. Histochemical application. *Acta physiol. scand.* 1957 *40*. 331 337
…, B. and F. Sjöqvist. Some principles about histochemistry of cholinesterase with special reference to the thiocholine method. *Bibl. anat.* (Basel) 1961 *2*. 1 10
…, O. P. and P. Gonick, Effects of phenoxybenzamine hydrochloride on canine lower urinary tract. *Urology* 1975. *6*. 323–330.
…, B. G. and J. S. Friedenwald, A histochemical method for localizing cholinesterase activity. *Proc. Soc. exp. Biol.* (N.Y.) 1949 *70* 617–622
McGuire, E. J., F. M. Wagner and R. M. Weiss, Treatment of autonomic dysreflexia with phenoxybenzamine. *J. Urol.* (Baltimore) 1976 *115* 53–55.
…, T. L., A. El Badawi, D. McDonald and E. Schenk, Innervation of the human urinary bladder. *Surg. Forum* 1966. *17* 505–506.
Nergårdh, A. and L. O. Boréus, Autonomic receptor function in the lower urinary tract of man and cat. *Scand. J. Urol. Nephrol.* 1972. *6*. 32 36.
Nergårdh, A. and J. Gerdin, Adrenergic receptor function in the urinary bladder. Observations in five children with myelomeningocele. *Scand. J. Urol. Nephrol.* 1974 *8* 114–119
…, M. M. Innervation of the bladder and urethra. *J. Anat.* 1969 *105* 210.
Owman, Ch. and N.-O. Sjöberg, The importance of short adrenergic neurons in the seminal emission mechanism of rat, guinea-pig and man. *J. Reprod. Fertil.* 1972. *28* 379–387
Owman, Ch., T. Owman and N.-O. Sjöberg, Short adrenergic neurons innervating the female urethra of the cat. *Experientia* (Basel) 1971. *27* 313–315.
Raezer, D. M., S. H. Greenberg, D. M. Jacobowitz, G. S. Benson, J. N. Corriere, Jr., and A. J. Wein, Innervation of trigonal area of canine urinary bladder. *Urology* 1976. *7* 349–375.
Raezer, D. M., A. J. Wein, D. Jacobowitz and J. N. Corriere, Jr., Autonomic innervation of canine urinary bladder. *Urology* 1973. *2*. 211 221
Schmitt, F. P. Fuchs and K. Stockamp. Estrogenic sensitivity of α-receptors in the urethra musculature. *Urol. int.* (Basel) 1976. *31* 13–19
…, B. *Distribution and interrelations of peripheral adrenergic and non-adrenergic nerve terminals.* M.D. Thesis Comm. Dept. Anat. Lund, Sweden 1970. No 5 1 51
Stockamp, K. and F. Schreiter, α-Adrenolytic treatment of the congenital neuropathic bladder. *Urol. int.* (Basel) 1975. *30*. 33.
Stewart, B. J., L. H. W. Banowsky and D. K. Montague, Stress incontinence: conservative therapy with sympathomimetic drugs. *J. Urol.* (Baltimore) 1976. *115* 558–559

SUNDIN, T and A. DAHLSTRÖM, The sympathetic innervation of the urinary bladder and urethra in the normal state and after parasympathetic denervation at the spinal root level. An experimental study in cats. *Scand J Urol Nephrol* 1973 7 131 149

SUNDIN T., A DAHLSTRÖM, L. NORLÉN and N SVEDMYR, The sympathetic innervation and adrenergic receptor activity of the human lower urinary tract in the normal state and after parasympathetic denervation. *Invest Urol.* 1976. In press.

TODD J K. and A. J MACK A study of human bladder detrusor muscle. *Brit J Urol.* 1969 41 448-459

ULMSTEN, U N-O SJÖBERG, P ALM, K E. ANDERSSON CH. OWMAN and B. WALLES. Functional role of an adrenergic sphincter in the female urethra of guinea pig. 1976. In manuscript.

WAKADE, A R and S N KIRPEKAR, Sympathetic innervation of urinary bladder of the guinea-pig. *Amer J Physiol* 1972. 223 1477–1480.

WHITFIELD H N P T DOYLE, M E. MAYO and N POOPALASINGHAM, The effect of adrenergic blocking drugs on outflow resistance. *Brit J Urol* 1976. 47 823–827

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Lower Body Negative Pressure and Effects of Autonomic Heart Blockade on Cardiovascular Responses

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Abstract

BJURSTEDT, H., G. ROSENHAMER and G. TYDÉN. *Lower body negative pressure and effects of autonomic heart blockade on cardiovascular responses.* Acta physiol. scand. 1977. 99. 353–360.

Heart rate, arterial pressure and cardiac output were recorded in eight healthy male volunteers during exposure to 80 mmHg of lower body negative pressure (LBNP) in the supine position before and after beta-adrenergic and combined beta-adrenergic and parasympathetic blockade of the heart as induced by the i[?] administration of propranolol 0.25 mg/kg b.wt. and atropine 0.04 mg/kg b.wt. After propranolol, heart rate response to LBNP averaged 48 of that observed without blockade indicating that LBNP-induced cardioacceleration of both sympathetic and parasympathetic origin. Tolerance to LBNP was reduced by beta-adrenergic blockade, since the decrease in mean arterial pressure during LBNP was exaggerated by such blockade. Although the addition of atropine markedly elevated mean arterial pressure and cardiac output in the control situation, tolerance to LBNP was not enhanced by this drug as judged from the arterial pressure response. Post LBNP overshoot in mean arterial pressure was strikingly augmented by combined cardiac effector blockade and was in part due to lingering elevation of total peripheral resistance, cardiac output remaining decreased for more than 110 after release of LBNP.

The arterial pressure response to a threefold increase of the gravitational force in the head–foot direction, associated with massive hypotension at head level and drainage of blood into the dependent part of the body, is not affected by beta-adrenergic blockade but is reduced after combined beta-adrenergic and parasympathetic blockade (Bjurstedt, Rosenhamer and Tydén 1974, 1976). However, the pressor response to a local decrease in the carotid sinus transmural pressure is not affected by autonomic heart blockade (Bjurstedt, Rosenhamer and Tydén 1975). It seemed of interest therefore to study the effects of autonomic heart blockade on the circulatory adaptation to lower body negative pressure (LBNP), since this maneuver may produce considerable pooling of blood in dependent veins with little if any effect on the arterial blood pressure at head level (for review see Wolthuis, Bergman and Nicogossian 1974). Accordingly we have investigated, in human subjects, the cardiovascular effects of 80 mmHg of LBNP before and during beta-adrenergic and combined beta-adrenergic and parasympathetic blockade as induced by the intravenous administration of propranolol and atropine.

Material and Methods

8 healthy well trained male volunteers served as subjects. Individual and d mensional data are pro Table I

The device used to produce LBNP consisted of a wooden box 130 50 36 cm with an elliptical ape at one end. A rubber seal was provided at the opening which fitted hermetically around the at t level of the iliac crest. The subject rested supine in the bo straddling a narrow padded saddle hich pro support during the suction exposures. The box was connected via a pressure regulator to a remotely lo preevacuated low pressure chamber which made possible the application of graded negative pre The time to reach the desired negative pressure was kept at about 30 seconds. A strain-gauge mano was used for monitoring the pressure in the box. The temperature inside the box as monitored tinuously nd was kept within ± 0.5°C.

Intraarterial pressure at heart level and heart rate were measured according to methods decribed where (Bjurstedt, Rosenhamer and Tydén 1974). Dichromatic earpiece densitometry was used for d mination of cardiac output This method, described Reed and Wood (1967), has shown satisfactory ment with results obtained with arterial cuvette densitometry (Reed and Wood 1967 Schneider Ch zadeh and Müller 1967 Wallgren 1975). The dichromatic earpiece (Waters XE 30.) was attached to the after rubbi g the pinna for 1 min with rubrifacient ointment (Transvasin®) and was secured to the ear dhesive tape Th ear and earpiece were then covered with green cloth to prevent influence of am light on the photocell. Calibration was achieved accordi g to the end-tail method (Beard and Wood 1!

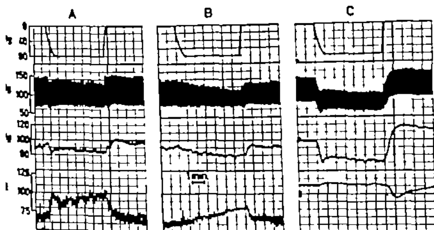
Design f experiment Prior to the actual expt ll subjects had been made f miliar with the subje sensations associated with LBNP The expt commenced with a 15 min period of rest in the supine pos in the box followed by a 4 mi exposure to 80 mmHg LBNP Beta-adrenergic blockade as then ach by the i v dministration of 0 25 mg/kg of propranolol over a 3 min period whereupon the 15 min re of rest and the 4 min exposure of 80 mmHg were repeated. Atropine (0.04 mg/kg) was then injected and the 15 min period of rest and 4 min period of LBNP were again repeated. determinations of ca output were made during the last 3 min of each 15 mi rest period and 3 consecutive determinations recorded during LBNP the f rst determination being made 20 s after attainment of 80 mmHg LBNP second after 1 min 50 s and the third after 3 min 20 s. Following the cessation of LBNP another two d minations of cardiac output were performed, the first after 20 s and the second after 1 min 50 s Heart arterial pressure and mean arterial pressure derived electronically were recorded continuously throu the expt.

Calculations. Heart rate and mea arterial pressure were averaged during the inscription of each i cator dilution curve In the resting condition the mean values of the two cardiac output determinations the correspond g values of heart rate and mean arterial pressure were calculated Total periphera sistance was calculated as mean arterial pressure (mmHg) divided by cardiac output (ml/s). The statis significance of differences between mea values were evaluated by pplying the t-test to the intraindiv differences (*cf* Fischer 1948).

TABLE I Functional and dimensional data.

Subject	Age years	Weight, kg	Height, cm	BSA m²	HR	MAP
JK	37	70	180	1 88	59	90
JT	.5	74	18	1 94	62	89
PH	29	88	184	2 10	47	9
MK	21	87	186	2.11	55	93
JP	27	63	173	1 75	54	89
LÖ	31	65	174	1 78	75	100
AA	26	85	190	1	55	81
FA	28	73	183	1 94	65	91

BSA = Body surface area from nomogram of Dubois and Dubois (1916). HR = Heart rate in beats after 15 min of supine rest. MAP = Mean arterial pressure in mmHg t heart level after 15 min of supine r



Segments of original recording showing time-dependent effects of LBNP (80 mmHg), on arterial re in the radial artery (AP), mean arterial pressure (MAP) obtained electronically from AP and heart before (A) and after beta-adrenergic (B) and combined beta-adrenergic and parasympathetic (C) ade

Results

1 shows an example of the circulatory changes produced by the application of 80 mmHg BNP before and after beta-adrenergic and combined beta-adrenergic and parasympa c blockade. Mean values of the observed responses are given in Table II–IV Although e of the subjects showed a progressive fall in mean arterial pressure during LBNP none rienced any symptoms of presyncopal nature.

Responses to 80 mmHg of LBNP before blockade With the application of negative pressure ke volume showed a progressive decrease (52% after 30 s, −60% after 3 min 20) reas heart rate gradually increased (41% and 66%, respectively). Cardiac output, efore, decreased initially by 33% but did not show any further reduction. Mean arterial sure decreased towards the end of the LBNP period (9% after 3 min 20), due to re of the total peripheral resistance to remain at the same high level throughout this od (30% after 20 s, 40% after 3 min 20 s).

After release of the negative pressure mean arterial pressure showed a slight, insignificant, rshoot accompanied by a moderate slowing of the heart rate (−8%, $p < 0.05$). Cardiac put remained decreased 20 s after cessation of LBNP (−11%, $p < 0.001$) as compared its value before negative pressure, whereas total peripheral resistance was increased 18% after 20).

Responses to 80 mmHg of LBNP after propranolol. After propranolol LBNP reduced an arterial pressure by 12% after 3 min 20 s, the absolute value being significantly lower n before blockade (78 mmHg and 83 mmHg, respectively $p < 0.05$). The LBNP-induced chyacceleration was markedly reduced (+16% after 20 s, +39% after 3 min 20 s) while reduction in stroke volume was much less evident than before propranolol. Cardiac tput was therefore reduced only by 18% after 20 s and by 24% after 3 min 20 s of LBNP

TABLE II Responses to 80 mmHg LBNP without blockade.

	Before LBNP	During LBNP			After LBNP	
		20 s	1 min 50 s	3 min 20 s	20 s	1 min 50 s
MAP	91±	89±2	86±3	83±3	94±3	93±
diff		−2±1	−5±2	−8±2	+3±3	+2±2
HR	59±3	83±4	92±4	98±5	60±5	54±3
diff		+24±4	+33±4	+39±5	+1±2	5±1
Q̇	8.1±0.4	5.4±0.4	5.4±0.4	5.4±0.4	7.2±0.3	7.7±0.3
diff		−2.7±0.1	−2.7±0.1	−2.7±0.1	−0.9±0.1	0.4±0.4
TPR	0.68±0.03	1.02±0.07	0.99±0.06	0.95±0.06	0.80±0.05	0.75±0.04
diff		+0.34±0.04	+0.31±0.03	+0.27±0.04	+0.12±0.03	+0.07±0.0[illegible]
SV	140±9	67±6	58±4	56±4	124±9	147±13
diff		−73±6	−8[illegible]±6	−84±7	−16±4	+7±9

MAP = mean arterial pressure, mmHg, HR = heart rate, beats/min, Q̇ = cardiac output, l/min, TPR peripheral resistance, mmHg s/ml, SV = stroke volume, ml. denotes statistical difference from co[illegible] value before LBNP − p 0.05 − p 0.01 − p 0.001

TABLE III Responses t[illegible] 80 mmHg LBNP after propranolol.

	Before LBNP	During LBNP			After LBNP	
		20	1 min 50 s	3 min 20 s	20 s	1 min 50 s
MAP	89±2	85±2	81±1	78±2	90±3	89±2
diff		−4±1	−8±2	−11±2	+1±1	0±1
HR	49±2	57±3	66±2	68±2	49±3	48±3
diff		+8±1	+17±2	+19±2	0±1	−1±1
Q̇	5.5±0.3	4.5±0.3	4.2±0.2	4.[illegible]±0.3	5.0±0.3	5.6±0.4
diff		−1.0±0.3	−1.3±0.2	−1.3±0.2	0.5±0.2	+0.1±0.2
TPR	1.00±0.07	1.17±0.10	1.20±0.08	1.16±0.4	1.11±0.10	1.00±0.08
diff		+0.17±0.06	+0.20±0.04	+0.16±0.07	+0.11±0.05	0.00±0.03
SV	116±10	81±7	64±5	63±6	107±11	120±14
diff		−35±6	−52±6	−53±6	−9±6	+4±8

Abbreviations as in T ble II

TABLE IV Responses to 80 mmHg LBNP after propranolol and atropine.

	Before LBNP	During LBNP			After LBNP	
		20 s	1 mi 50 s	3 mi 20 s	[illegible]0	1 min 50 s
MAP	100±3	85±2	84±2	79±2	111±5	117±4
diff		−15±3	−16±3	−21±3	+11±3	17±3
HR	91±4	95±3	96±3	96±3	82±4	88±4
diff		+4±2	+5±	+5±2	9±1	3±1
Q̇	7.6±0.6	4.0±0.3	4.0±0.3	4.1±0.3	6.5±0.5	7.3±0.5
diff		−3.6±0.4	−3.6±0.4	−3.5±0.5	1.1±0.3	0.3±0.2
TPR	0.82±0.07	1.32±0.10	1.31±0.10	1.20±0.11	1.08±0.11	0.98±0.07
diff		+0.50±0.06	+0.49±0.05	+0.38±0.08	+0.25±0.08	0.16±0.08
SV	86±8	43±4	42±4	43±4	81±9	86±7
diff		−43±5	−44±5	43±6	−5±2	0±

Abbreviations as i T ble II

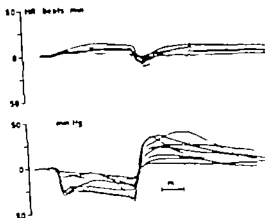


Fig. 2. Individual responses in heart rate (HR) and mean arterial pressure (MAP) during and after 4 min of LBNP after combined ... effector blockade, redrawn from ... recordings (all pre-LBNP values set to zero). Note residual changes in heart rate ... of full blocking doses of propranolol and atropine, also remarkably strong and long lasting post-LBNP overshoots in mean ... pressure

its absolute value, however being significantly less than before blockade. The maximal increase in calculated total peripheral resistance was 20%.

When pressure in the box was returned to ambient, cardiac output and total peripheral resistance remained temporarily somewhat decreased and increased, respectively as compared to pre-LBNP values whereas mean arterial pressure and heart rate rapidly returned to normal

Responses to 80 mmHg or LBNP after propranolol and atropine After combined beta adrenergic and parasympathetic blockade LBNP produced a marked fall in mean arterial pressure (15% after 20 s and −21% after 3 min 20 s). However the absolute value after 3 min 20 s was not significantly lower than with propranolol alone. Heart rate increased by 5 (p 0.05). Stroke volume decreased by 50% within 20 s and did not show any further reduction, its absolute value being significantly lower than during LBNP with no blockade or with propranolol alone. Cardiac output was reduced by no less than 47% already after 20 s. Calculated total peripheral resistance therefore showed a marked increase (61% after 20 s, 46 after 3 min 20 s).

In the recovery period after LBNP following autonomic heart blockade, mean arterial pressure showed marked and prolonged overshoots in some individuals (Fig. 2). For the group, the overshoot was 11% after 20 s and 17% after 1 min 50 s. The overshoot was associated with a clearcut slowing of the heart rate. As was the case with no drug and with propranolol alone cardiac output remained decreased for at least 1 min 50 s. The return of total peripheral resistance towards normal was remarkably sluggish, this variable still being elevated by 20% after 1 min 50 s as compared to its pre-LBNP value.

Discussion

Lower body negative pressure is a powerful tool for altering cardiovascular dynamics and has been shown to produce many of the same changes in cardiac output, heart rate and arterial pressure that occur with upright tilting. Indeed, most previous investigators of the effects of LBNP have been interested in pressure ranges that produce physiological changes

comparable to those observed during orthostasis and 70° head-up tilt, *i.e* 40-50 mmH (for review see Wolthuis, Bergman and Nicogossian 1974). For this reason, information i scarce as to the effects of LBNP of greater magnitude. Furthermore, it seems that only few studies have included measurements of cardiac output intraarterial pressure and hea rate during LBNP (Stevens and Lamb 1965 Murray *et al* 1968 Rowell *et al* 1972).

In the control situation in the present experiment LBNP produced a drastic reduction i stroke volume (52 % after 20 s, 60 % after 3 min 20 s) accompanied by a clearcut increase i heart rate (41 % after 20 s, 66 % after 3 min 20 s) These findings are in agreement with tho of Stevens and Lamb (1965) who used the same level of negative pressure. Stevens and Lam extended the LBNP periods until signs of presyncope occurred and found that the subjec could be divided into symptomatics and asymptomatics *i.e* those who experienced pr syncopal symtoms within 5 min and those who did not. It was noted that asymptoma subjects did not show any decrease in mean arterial pressure during 5–3 min of exposure 80 mmHg while symptomatics did. Although the present LBNP exposures were not extend beyond 4 min they support the findings of Stevens and Lamb since it was found that so subjects tolerated the exposure well while others showed a slight but continuous fall in me arterial pressure during LBNP In the latter case the fall in blood pressure was due to failure to maintain total peripheral resistance throughout the LBNP period while cardi output was well maintained

After propranolol tolerance to LBNP was reduced as judged from the blood press response *i e* mean arterial pressure was significantly lower after 3 min 20 s as compared the situation before blockade. This can be explained by the negative chronotropic effe of propranolol and the resulting reduction in cardiac output Although there was a mo potent vasoconstrictor response to LBNP after propranolol it was not sufficient to maint arterial pressure at the same level as before blockade This finding is notable since the aggerated curtailment of cardiac output occurring during head-up tilt (Sannerstedt, Joh and Conway 1970 Loeppky 1975) or exposure to increased gravitational force (Bjurst *et al* 1974) after propranolol is well compensated by a concomitant rise in peripheral sistance. In the latter cases the carotid sinus hypotension associated with head-up tilt a increased gravitational force may initiate a stronger vasoconstrictor reflex than does LBN

After propranolol, heart rate response to LBNP was only 48 % of that observed befo blockade, the absolute values after 3 min 20 s being 68 beats/min and 98 beats/min, resp tively This finding implies that the heart rate response to LBNP is of both sympathetic a parasympathetic origin It also seems probable that vagal withdrawal was far from maxim during LBNP since heart rate at the end of the LBNP period after propranolol (68 bea min) was much lower than that obtaining after propranolol and atropine (91 beats/min rest)

With combined cardiac effector blockade heart rate, cardiac output and mean arte pressure increased considerably these findings being concordant with other expts. concern autonomic heart blockade in the recumbent position (Julius *et al* 1971 Nordenfelt 19 Ellis and Julius 1973 Korner *et al* 1973 Bjurstedt *et al* 1975) However following appli tion of LBNP there was a drastic reduction in cardiac output and mean arterial pressure the same levels as obtained during LBNP with propranolol only These findings favor t

cept that atropine does not enhance the tolerance to hydrostatically induced blood volume placement as brought about by LBNP (Murray and Shropshire 1970), head-up tilt (Sieker, Leonard and Warren 1957) or increased gravitational force (Bjurstedt *et al.* 1976). At the termination of negative pressure before blockade there was a small overshoot in mean arterial pressure which was found to be due to a persisting increase in total peripheral resistance, cardiac output remaining temporarily suppressed. This is in accordance with the findings of a sustained increase in vasoconstrictor tone after the Valsalva maneuver (*cf* Sharpey-Schafer 1963), this maneuver producing similar changes in venous return as LBNP. There is also a direct correlation between the rise in peripheral resistance during the Valsalva maneuver and the magnitude of the ensuing overshoot in mean arterial pressure (Sharpey-Schafer 1963). The finding that the LBNP-induced increase in total peripheral resistance was markedly enhanced by combined blockade therefore at least partly explains the remarkable overshoot in mean arterial pressure after propranolol and atropine (see Fig. 2). A contributing factor may have been blockade of beta receptor mediated vasodilatation (*cf* Brick *et al.* 1966, Mahon 1966).

Concomitantly with the overshoot in mean arterial pressure a slowing of the heart was observed, the magnitude of which was directly related to the intensity of the overshoot. This slowing of the heart rate is probably the result of reflexes originating in arterial stretch receptors responding to the increase in arterial pressure. Involvement of low pressure receptors is less likely since the effect of increased venous return on intrathoracic low pressure receptors is a speeding rather than a slowing of the heart rate (Bainbridge 1915, Linden 1972). The fact that the post-LBNP bradycardia was not abolished by the combined administration of atropine and propranolol is notable since no slowing of the heart rate occurs in the denervated heart following the Valsalva maneuver or a change from erect to recumbent posture (Beck, Barnard and Schrire 1969, Stinson *et al.* 1970). It can be assumed therefore that the overshoot in arterial pressure exerted a reflex influence on the cardioinhibitory center of such a magnitude as to make the cardiac effector blockade incomplete even though the doses of atropine and propranolol used in the present expts. should normally produce an almost complete sympathetic and parasympathetic "isolation" of the heart (*cf* Jose 1966).

References

BAINBRIDGE, F. A., The influence of venous filling upon the rate of the heart. *J. Physiol.* (Lond.) 1915. 50 65–84.

BANG, E. F. and E. H. WOOD, Estimation of cardiac output by the dye dilution method with an ear oximeter. *J. appl. Physiol.* 1951. 4 177–187.

BECK, W., C. N. BARNARD and V. SCHRIRE, Heart rate after cardiac transplantation. *Circulation* 1969. 40. 437–445.

BJURSTEDT, H., G. ROSENHAMER and G. TYDÉN, Acceleration stress and effects of propranolol on cardiovascular responses. *Acta physiol. scand.* 1974. 90. 491–500.

BJURSTEDT, H., G. ROSENHAMER and G. TYDÉN, Cardiovascular responses to changes in carotid sinus transmural pressure in man. *Acta physiol. scand.* 1975. 94 497–505.

BJURSTEDT, H., G. ROSENHAMER and G. TYDÉN, Gravitational stress and autonomic cardiac blockade. *Acta physiol. scand.* 1976. 96. 526–531.

BRICK, I., W. E. GLOVER, K. J. HUTCHISON and I. C. RODDIE, Effects of propranolol on peripheral vessels in man. *Amer. J. Cardiol.* 1966. 18 329–332.

DUBOIS, D and E. F DUBOIS, A formula to estimate the approximate surface area if height and weight be known. *Arch Intern. Med* 1916. *17* 862–871

ELLIS, C. N and S JULIUS, Role of central blood volume in hyperkinetic borderline hypertension. *Br Heart J* 1973 *35* 450–455

FISHER, R. A *Statistical Method for Research Workers* 10th Ed. Edinburgh, Oliver and Boyd Ltd 1948 pp 354

JOSE, A. D Effect of combined sympathetic and parasympathetic blockade on heart rate and cardiac function in man. *Amer J Cardiol.* 1966. *18* 476–478

JULIUS, S., A. V PASCUAL, R. SANNERSTEDT and C. MITCHELL, Relationship between cardiac output and peripheral resistance in borderline hypertension. *Circulation* 1971 *43* 382 390.

KORNER, P I J SHAW J B UTHER, M J WEST R. J MCRITCHIE and J G RICHARDS, Autonomic and non-autonomic circulatory components in essential hypertension in man. *Circulation* 1973. *48*. 107–112.

LINDEN R J Function of nerves of the heart. *Cardiovasc. Res.* 1972. *6*. 605–626.

LOEPPKY J A. Cardiorespiratory responses to orthostasis and the effects of propranolol *Aviat. Space Environ. Med.* 1975 *46* 1164–1169

MAHON, W A., The peripheral vascular effects of beta adrenergic blocking agents in man. *Angiology* 1966. *3* 304–317

MURRAY R. H and S. SHROPSHIRE, Effect f atropine on circulatory responses to lower body negative pressure and vasodepressor syncope *Aerospace Med.* 1970. *41* 717 722.

MURRAY R H L. J THOMPSON J A. BOWERS and C. D ALBRIGHT Hemodynamic effects of graded hypovolemia and vasodepressor syncope induced by lower body negative pressure. *Amer Heart J* 1968 *76* 799–811

NORDENFELT I Haemodynamic response to exercise after combined sympathetic and parasympathetic blockade of the heart. *Cardiovasc Res* 1971 *5* 215–222.

REED J H Jr and E. H WOOD, Use of dichromatic earpiece densitometry for determination of cardiac output. *J appl Physiol* 1967 *23* 373–380

ROWELL, L. B J M R. DETRY J R BLACKMON and C. WYSS, Importance of the splanchnic vascular bed i human blood pressure regulation. *J appl Physiol* 1972. *32* 213–220

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SCHNEIDER, K. W von, A. CHEIKHZADEH und W MÜLLER, Die Bestimmung des Herzminutenvolumens mit einem dichromatischen Ohrdensitometer und vereinfachter Berechnung durch Computer *Cardiology* (Basel) 1967 *51* 349–358

SHARPEY-SHAFER, E. P Effect of respiratory acts on the circulation. In *Handbook of Physiology* Circulation III Washi gton, D C. Am. Physiol. Soc. 1965 1875–1886.

STEVENS, P M nd L. E. LAMB, Effects of lower body negative pressure on the cardiovascular system *Amer J Cardiol* 1965 *16* 506–515

STINSON, E. B. R. B GRIEPP D A CLARK, E. DONG and N E. SHUMWAY Cardiac transplantation in man *J thorac cardiovasc Surg* 1970. *60* 303–3 1

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WEISSLER, A. M J J LEONARD and J V WARREN, Effects of posture and atropine on the cardiac output. *J clin Invest* 1957 *36* 1656–1662.

WOLTHUIS, R. A. S. A. BERGMAN and A. E. NICOGOSSIAN Physiological effects of locally applied reduced pressure in man. *Physiol Rev* 1974 *54* 566–595

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By

TOM BRISMAR

Received 15 September 1976

Abstract

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The present investigation was performed in order to find out whether the action of Ca is slow or rapid at very rapid changes between external solutions of different [Ca]s. Techniques for rapid switches between solutions with different compositions have been used for the isolated myelinated nerve fibre. Thus Stämpfli (1958) has used a small stop cock combined with a small dead space in order to obtain rapid switches. Vierhaus and Ulbricht (1971) have measured the rate of such rapid switches through observations of the changes in Na currents during switches between solutions with different [Na]s. They found on fibres from *Rana esculenta* that the change in external [Na] followed an exponential time course with a time constant of 30–50 ms. In order to press the rate for such a solution switch still further the present experiments on *Xenopus* fibres were performed with a dead space minimized to 0.02 mm³. In spite of that a 50% complete solution switch required 100–150 ms, as judged from the changes in I_{Na} (Na-current) at a rapid switch between two solutions with different [Na]s. The half time for the effect of rapid changes in [Ca] was twice as long or 200–300 ms. The difference in rate of action between Na and Ca will be referred to the ultrastructure of the node region and the properties of the node gap substance.

Methods

Potential clamp experiments were performed on single myelinated nerve fibres isolated from the sciatic nerve of the frog (*Xenopus laevis*). The potential clamp technique was essentially similar to the one developed by Dodge and Frankenhaeuser (1958). A rapid switch from one solution to another one was obtained by letting an amount of new solution, which was initially held together by surface tension as isolated from the pool containing the node under observation suddenly fuse with the pool and flow past the node. The dead space in the pool upstreams the node was kept at a practical minimum of about 0.02 mm³. The solution flow was then kept unidirectional past the node in order to prevent back flow of the old solution. The time for contact made between the two solutions was monitored electrically. The rate of change in ionic concentration at the axon membrane surface was measured from the change in the peak amplitude of the inward membrane current during short test pulses repeated every 50 ms. The half time of the solution changes was estimated from records of membrane current in the individual node tested with a half-and-half mixture of the basic solutions. The membrane was polarized to −120 mV between the test pulses. The polarization removed the Na inactivation caused by the test pulses. The composition of the test solutions is described in Table I. All solutions were buffered with 2.5 mM Tris to pH 7.4 at 25°C.

Results

Fig. 1A is a plot of the peak membrane current (mainly carried by Na) associated with short lasting test steps to −30 mV with a conditioning membrane polarization to −120 mV. The [Ca] was held at 0.27 mM throughout while the [Na] was 25 mM before the solution switch and 115 mM after the switch. The Na permeability (P_{Na}) was nearly fully turned on

TABLE I. Composition of test solutions (mM)

No	1	2	3	4	5
NaCl	115	115	25	25	11
$CaCl_2$	14.8	0.27	14.8	0.27	[illegible]
KCl	—	—	90	90	2.5
Tris	2.5	2.5	2.5	2.5	2.5

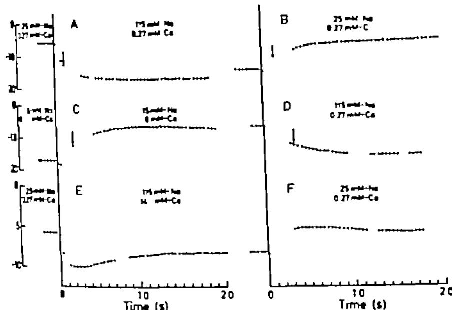


Fig. 1. Changes in total membrane current associated with rapid changes in ionic composition of external solution (indicated). Repeated positive potential pulses to −30 mV from continuous polarization to −120 mV between test pulses. Duration of test pulses 5 ms. Repetition rate 23 per s. Beginning of solution changes indicated by the vertical lines and by arrows at the half-time of the solution changes. Temperature 9 C.

of the test pulse with the fibre in these solutions. The plot ought therefore to indicate the rate of change of [Na] immediately outside the membrane. Thus the concentration change was half way complete in about 150 ms. The switch back to low [Na] was similarly 50 % complete within about 150 ms (Fig. 1 B).

Rapid changes in [Ca] were performed in a similar way. The potential of the test pulse was then adjusted to the potential region where P_{Na} was most affected by changes in [Ca]. Fig. 1 C and D show the time course of the change in I_{Na} peak amplitude during [Ca] changes. The membrane potential during the test pulse was −30 mV. The Na permeability mechanism was almost fully turned on in 0.27 mM-Ca whereas it was only partially so in 14.8 mM Ca. The change in amplitude of I_{Na} therefore indicated the change in the P_{Na}-potential relation caused by the change in [Ca] of the bulk of the external solution. The half-time of this effect of [Ca] changes was 200–300 ms or about twice as long as that of [Na] changes. In a similar experiment on another fibre the half-time for the [Na] effect was 100 ms when going from low [Na] to high as well as when switched back to low [Na]. The half-time for the effect of [Ca] changes was about 300 ms, i.e. about three times as long.

A high repetition rate of test pulses (20 pulses per s) was required to obtain a resolution of the time course of the I_{Na} changes. A slow decrease in peak P_{Na} appeared at this high repetition rate. This has recently been shown to be related to a slow inactivation process (Fox 1975; Brismar 1976). These changes introduced an uncertainty in the judgement of the estimate of the peak P_{Na} amplitude corresponding to the half-time of the ionic concentration

change. The difference between the half time at the change from low to high [Ca] and the half time from high to low [Ca] in Fig. 1 C and D might consequently be apparent only.

A slower rate of change of the peak I_{Na} associated with the change in [Ca] compared to that associated with the change in [Na] was further observed when simultaneous changes of [Na] and [Ca] were performed. Fig. 1 E shows a change to high concentrations and Fig. 1F, the reverse change. The amplitude of the initial current depended on the sequence of solution change. The peak I_{Na} was larger soon after the change from low [Ca] and low [Na] to high [Ca] and high [Na] than in the steady state in any of these solutions. An over-shoot of this magnitude was not seen at the solution change in the reverse sequence. It is quite possible that an application of a mixture of 2 solutions as used in these expts. is as-sociated with I_{Na} of greater amplitude than is obtained in each of the basic solutions. In that case, however transiently larger membrane currents should be recorded in both solution switches. By exclusion it is therefore concluded that the transient increase in the initial current shown in Fig. 1E must depend on a slower effect of [Ca]-than of [Na]-exchange on the membrane. At the first solution change there was a transient increase in the peak I_{Na} when the high [Na] reached the membrane followed by the turn off of P_{Na} associated to the slower effect of high [Ca]. At the second solution change there was a rapid decrease in [Na] and in the size of the inward current followed by a slower increase in the P_{Na} caused by the low [Ca].

In all fibres (about 20) tested in the way described by Fig. 1E and F this sequence of events was observed. The potential of the test pulse, the [Ca]:s and the [Na]:s of the test solutions influenced the magnitude of the overshoot. When the solution changes were per-formed with repeated application of solutions with identical compositions no change oc-curred in the size of the initial current. The pulse duration was short in these expts. A small continuous increase in the inactivation of the Na permeability mechanism could anyhow not be avoided. This effect developed in low [Ca]. The inactivation thus affected the ampli-tude of the initial current but in a direction opposite to that caused by the rapid solution changes.

Discussion

Potential clamp expts. were performed on single myelinated nerve fibres of *Xenopus laevis*. Short positive potential pulses were repeated with a frequency of 20 Hz while the amplitude of the associated membrane current was recorded. The size of the inward Na-current depends on the Na-permeability of the membrane and the electrochemical driving force for Na. Rapid changes in the external ionic composition were performed by rapid switches between solutions of different salt concentrations. The advantage of the described method for solu-tion changes was that the dead space was minimal and that a distinct indication of the be-ginning of the solution change was obtained at the electric contact between the old and the new solution. The half time of the change in Na-current was estimated to be 100–150 ms at changes between solutions of different [Na]:s. On *Rana esculenta* Vierhaus and Ulbricht (1971) found with corresponding solution switches that the time constant of the exponential time course of the Na-current change was 30–50 ms, *i.e.* the half-time was 20–35 ms. The discrepancy between the two experimental measurements may be due to a species difference between fibres from *Rana* and *Xenopus* respectively

An approximative estimate of the conditions for the solution change reveals that a volume) times larger than the dead space (0.02 mm³) flows past the fibre in about 20 ms. The new ution ought thus to have reached the fibre within this time. Excessive eddy currents might some extent slow down the rate of solution change in the immediate vicinity of the fibre. he effects of the solution switches were clearly slower than accountable to the solution ange. The ionic transport from an unstirred layer outside the fibre to the nodal membrane most likely due to diffusion. Uncomplicated free diffusion in a plane sheath of 10 μm ickness would at a step concentration change at one surface change the concentration at e other surface (impermeable) to half complete in about 40 ms. The sheath thickness was mewhat arbitrarily taken as 10 μm (6 μm nodal cement and 4 μm thickness of unstirred yer). The effects measured were clearly slower. However, the assumption of uncomplicated ee diffusion might not hold for the structure outside the nodal membrane.

Investigations of the ultrastructure of the nodal region in frog fibres (Robertson 1959) and ventral foot fibres of the cat (Berthold 1968) show that the axonal membrane is covered by several structures in the nodal gap. These investigators further observed that the nodal gap was especially deep and narrow in large fibres as were used in the present study. This means that the free path for ions diffusing through the nodal gap can be very narrow, at its narrowest part in the gap about 0.02 of the nodal area. This will obviously impede the ionic flux between the membrane and the bulk of the external solution. It further explains why the half-time for the effect of [Na] changes is 100–150 ms instead of 40 ms as was theoretically calculated for the diffusion through the nodal gap.

The half-time of the effect of changes between solutions of different [Ca] was 200–300 ms, which is twice that of the half-time of the effect of [Na] changes. This difference is obviously not explained by the difference between the diffusion coefficients which is much less. It is, however, known that the nodal space, described by Hess and Young (1952) as the cementing disc, contains a substance, with the histochemical staining properties of a sulphated mucopolysaccharide (Langley and Landon 1967). These mucopolysaccharides have a large cation binding capacity (Langley 1969, 1970, Landon and Langley 1971). The combination of a limited diffusion and a large cation space in the nodal gap will limit the rate of change in cation concentration at the axonal membrane. This would also decrease the rate of [Ca] changes more than the rate of [Na] changes since the affinity for Ca is larger than for Na in polyanions containing both sulphate and carboxyl residues (Scott 1967, Langley 1970). The concentration of negative groups in the nodal gap substance is unknown. In the mucopolysaccharide matrix of human cartilage it was estimated by Maroudas (1968) to 0.2 Eqv/l. A similar charge density in the nodal gap substance would by electrostatic binding of Ca considerably increase the effective Ca-space compared to that indicated by the volume of the nodal space. Except for mucopolysaccharides the nodal gap contains numerous villi- or finger-like processes extending perpendicular to the axon surface from the Schwann cells bordering the nodal gap. The membrane surface of these processes may also be negatively charged and thus contribute to an absorption of cations (polyvalent in particular) in the nodal gap. Due to a Ca-absorption in the nodal gap the local Ca content may be considerably higher than in the external solution. It should, however, be pointed out that Ca ions absorbed in this way are immobilized and should not effect the permeability properties of the axonal membrane.

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During recent years several investigators have given evidence which supports the hypothesis that the mechanism for Ca-action is a neutralization of negative charges fixed on the surface of the axonal membrane. Such a neutralization could simply be due to the screening effect of a diffuse double layer of cations or in addition, to a specific Ca-binding. It is likely that the accumulation of Ca in the double layer would be rapid (see Grahame 1947) relative to the rates observed in the present investigation.

It is concluded that the slow action of Ca observed in the present investigation probably is related to the properties of the nodal gap. Especially important for the ionic exchange between the external solution and the axonal membrane is the cation binding property which has been ascribed to the nodal gap substance.

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References

BERTHOLD, C. H. Ultrastructure of the node paranode region of mature feline central lumbar spinal-root fibres. *Acta Soc. Med. upsalien.* 1968. *73* Suppl. 9

BREMAR, T. Effects of ionic concentration on permeability properties of nodal membrane in myelinated nerve fibres of *Xenopus laevis*. Potential clamp experiments. *Acta physiol. scand.* 1973. *87* 474–484

BREMAR, T. Processes for sodium permeability inactivation in the myelinated nerve fibre of the frog (*Xenopus laevis*). *Acta physiol. scand.* 1976. *97* 258–260.

BREMAR, T. and B. FRANKENHAEUSER, The effect of calcium on the potassium permeability in the myelinated nerve fibre of *Xenopus laevis*. *Acta physiol. scand.* 1972. *85* 237–41

BREMAR, T. and B. FRANKENHAEUSER, Effects of ionic concentration on sodium permeability properties of myelinated nerve fibres of *Xenopus laevis*. *J. Physiol.* (Lond.) 1975 *249* 549–559

D'ARRIGO, J. S., Possible screening of surface charges on crayfish axons by polyvalent metal ions. *J. Physiol.* (Lond.) 1973 *231* 117–128.

D'ARRIGO, J. S. Axonal surface charges: binding screening by divalent cations governed by external pH. *J. Physiol.* (Lond.) 1974 *243* 757–764

D'ARRIGO, J. S., Axonal surface charges: evidence for phosphate structure. *J. membr. Biol.* 1975. *2*. 253–263

DODGE, F. A. and B. FRANKENHAEUSER, Membrane currents in isolated frog nerve fibre under voltage clamp conditions. *J. Physiol.* (Lond.) 1958 *143* 76–90

EHRENSTEIN, G. and D. L. GILBERT. Evidence for membrane surface charge from measurement of potassium kinetics as a function of external divalent cation concentration. *Biophys. J.* 1973. *13* 495–497

FOX, J. M., Slow variations of the sodium conductivity in the membrane of myelinated nerve fibres. *Pflügers Arch. ges. Physiol.* 1975 *355* R69

FRANKENHAEUSER, B. and A. L. HODGKIN. The action of calcium on the electrical properties of squid axons. *J. Physiol.* (Lond.) 1957 *137* 18–244

GRAHAME, D. C., The electrical double layer and the theory of electrocapillarity. *Chem. Rev.* 1947 *41* 441–501

HARTZ, T. and W. ULBRICHT. Comparison of the effects of calcium and lanthanum on the crayfish giant axon. *Pflügers Arch. ges. Physiol.* 1973. *345* 281–294

HESS, A. and J. Z. YOUNG, The nodes of Ranvier. *Proc. R. Soc. B* 1952. *140* 301–320

HILLE, B., Pharmacological modifications of the sodium channels of frog nerve. *J. gen. Physiol.* 1968 *51* 199–219

LANDON, D. N. and O. K. LANGLEY. The local chemical environment of nodes of Ranvier: a study of cation binding. *J. Anat.* 1971 *108* 419–432.

LANGLEY, O. K. Ion-exchange at the node of Ranvier. *Histochem. J.* 1969 *1* 295–309

LANGLEY, O. K., The interaction between peripheral nerve polyanions and alcian blue. *J. Neurochem.* 1970. *17* 1535–1541

LANGLEY, O. K. and D. N. LANDON, A light and electron histochemical approach to the node of Ranvier and myelin of peripheral nerve fibres. *J. Histochem. Cytochem.* 1967 *15*. 722–731

[illegible], A., Physicochemical properties of cartilage in the light of ion exchange theory. *Biophys. J.* 1968 8 575–595
[illegible], G. N. and A. P. NAUMOV. Effect of surface charge on the steady-state potassium conductance of nodal membrane. *Nature* (Lond.) 1970. 228. 164–165.
[illegible], J. D. Preliminary observations on the ultrastructure of nodes of RANVIER. *Z. Zellforsch.* 1959 50 533–540
[illegible], C. L., The interactions of calcium [illegible] *Myxicola* giant axons and [illegible] description in terms of simple surface charge model. *J. Physiol.* (Lond.) 1975 248. 613–6[illegible]4.
[illegible], J. E., Ion binding in solutions containing acid mucopolysaccharides. In *The Chemical Physiology of Mucopolysaccharides.* Editor G. QUINTARELLI. Little, Brown, Boston. 1967 pp. 171–187
[illegible], R., Die Strom-Spannungs-Charakteristik der erregbaren Membran eines einzelnen Schnürrings und ihre Abhängigkeit von der Ionenkonzentration. *Helv. physiol. pharmacol. Acta* 1958 16. 127–145
[illegible], J. and W. ULBRICHT, Effect of [illegible] sudden change in sodium concentration on repetitively evoked action potentials of single nodes of RANVIER. *Pflügers Arch. ges. Physiol.* 1971 326 76–87
[illegible], W. Calcium and lanthanum effects at the nodal membrane. *Pflügers Arch. ges. Physiol.* 1974 350. [illegible]–[illegible].

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The Effects of Variations in Extracellular Magnesium Concentration on Electrical and Mechanical Activity in Rat Portal Vein

By

STEFAN B SIGURDSSON and BENGT UVELIUS

Received 22 September 1976

Abstract

SIGURDSSON S B and B UVELIUS. *The effects of variations in extracellular magnesi concentration on electrical and mechanical activity in rat portal vein.* Acta physiol sca 1977 99 368–376.

The effects of various concentrations of extracellular Mg^{2+} on electrical nd mechanical activity of the portal vei were studied The ntegrated spontaneous contractile activity n the preparation was large Mg^{2+}-free solution, decreased t about 50 % at 1 2 mM Mg^{2+} and was almost completely abolished at 10 Mg^{2+} Each spontaneous contraction became smaller whereas contraction frequency was less affected Suc gap recordings showed that the reduced pontaneous mechanical output was associated ith decreased ele cal activity on increasing $[Mg^{2+}]_o$. Increasing $[K^+]_o$ from 6 t 12 mM normalized the spontaneous mecha activity in 10 mM Mg^{2+} solutio Local registration of electrical activity with 3 extracellular glass capi electrodes showed that inactive areas developed t the h gh Mg^{2+} concentrations. These findings ind that Mg^{2+} exerts a hyperpolarizing action o the smooth muscle cell membrane and, at the highest c centration, terferes with intercellular propagation. $[Mg^{2+}]_o$ i the range of 0–10 mM had no effect on amplitude of K (122 mM) contractures t $[Ca^{2+}]_o > 0.5$ mM At $[Ca^{2+}]_o < 0.5$ the amplitude decre with increasing $[Mg^{2+}]_o$. The latter observation ndicates that Mg^{2} ca interfere with the Ca^{2}-per bility of the depolarized cell membrane. 1 mM EDTA Na tris 0 mM Mg^{2+} nd 0 mM Ca^{2+} seems lower intracellular Mg^{2+} below the minimum concentratio needed for contractile activity

The extracellular magnesium concentration which may vary considerably under patholog conditions (see *e.g* Walser 1967) affects blood flow resistance through a direct effect vascular smooth muscle (Viveros and Somjen 1968) It was suggested by Altura and Al (1974) that Mg-ions can affect the excitation–contraction coupling in vascular smo muscle by participation in the regulation of the cell membrane permeability and bin capacity of calcium. It has been demonstrated that isolated actomyosin from smooth mu is dependent on Mg ions for ATPase activity (Murphy *et al* 1969). Magnesium play fundamental role in many biochemical reactions including the release and transfer of ph phate groups (see *e.g* Livingstone and Wacker 1971)

The aim of the present study was to obtain further informatio on the effect of variati

f extracellular magnesium concentrations on smooth muscle by recording the electrical and mechanical activity in the isolated rat portal ein. An attempt was also made to lower tracellular Mg^{++} by chelating the ion with EDTA.

A preliminary report of the present study has been published earlier (Sigurdsson and Johansson 1975).

Material and Methods

About 40 portal vein preparations from rats of the Sprague-Dawley strain weighing 200 to 300 g were used. The animal was killed by blow on the neck and the vein was dissected out. The preparations used in experiments where only isometric tension was recorded were about 0.5 cm long. They were mounted in 4 ml muscle organ bath between muscle holder and force transducer (Grass FT03). The muscles were stretched to and maintained at preload of about 4 10^{-4} N and allowed to accommodate for at least 1.2 h before the beginning of the expts. For simultaneous recording of electrical and mechanical activity about 2 cm of the portal-mesenteric vein as removed and mounted in sucrose gap apparatus as described by Axelsson et al. (1967). The electrical signals were amplified and recorded in an AC mode and the number of spikes counted electronically as described by Johansson and Mellander (1975).

For simultaneous recording of electrical activity from three different sites on the muscle, bout 1 cm of the vein was dissected out and cut open longitudinally. It was stretched flat between muscle holder and force transducer (Grass FT03) at preload level of 4 10^{-4} N. Three glass capillary electrodes containing physiological salt solution are then placed on different sites of the preparation (for further details see Ljung and Stage 1970). The AC coupled signals were amplified and recorded on the Grass polygraph.

The bathing media in the expts. were based on Tris buffered solution of the following composition in mM: NaCl 120, KCl 6, glucose 11.5 and tris (hydroxymethyl) aminomethane (Trizma base, Sigma Chemical Co) 30. This solution was titrated ith HCl to pH of 7.4 at 37 C, and it will in the following be referred to "Na tris". $CaCl_2$ and $MgCl_2$ are then added to this solution to obtain desired concentrations. The accommodation solution was Na-tris with 2.5 mM Ca^{++} and 1.2 mM Mg^{+} I K-tris" solution all NaCl was replaced ith equimolar amounts of KCL 1 mM EDTA was used to get solution low (10^{-8} M) in (Hubbard et al. 1968). All solutions were bubbled ith 100 O_2.

In order to avoid the possible influence of noradrenaline release from the adrenergic nerve supply by these ion changes, phenoxybenzamine (Dibenzyline, Smith, Kline & French, generously supplied by Rosen & Co, Malmö, Sweden) was added to the accommodation solution to concentration of 10^{-6} M so that no response to added noradrenaline was obtained.

Results

The effect of Mg^{++} on the spontaneous mechanical activity of the rat portal vein was studied. The spontaneous phasic contractions in Na-tris with 2.5 mM Ca^{++} and 0 Mg^{+} are seen in Fig. 1 A. Fig. 1 B–E shows the results of increasing the Mg^{++}-concentration, in steps from 1.2 mM (B) to 10 mM (E). The muscle had been in the respective solution for 1/2 h when these recordings were obtained. It is clearly seen that the amplitude of the phasic contractions is diminished considerably by increasing Mg^{+} whereas the contraction frequency is almost unchanged. This effect was not due to changes in osmolality as osmotic compensation by adding sucrose to the 0 Mg^{++} solution or reducing NaCl in the Mg-high solutions did not affect the response.

Fig. 2 shows simultaneous recordings of mechanical and electrical activity in the sucrose gap apparatus. The recordings are from top to bottom, isometric active force, integrated active force sampled over 1 min periods, number of spikes, and AC-recording of electrical activity. Panels 2 A–D show the results of increasing the Mg^{+}-concentration from 0 to 10 mM. The contraction amplitude diminishes as in Fig. 1 and, concomitant with this, a

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Received 2. September 1976

Abstract

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The aim of the present study was to obtain further information on the effect of variations

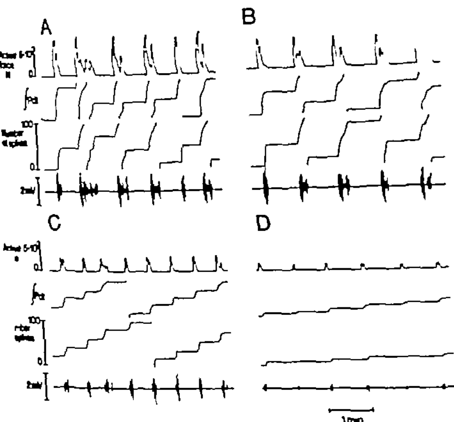


Fig. 2 Simultaneous recording of mechanical and electrical activity. The tracings from top to bottom represent active force, integrated active force, number of electrical spikes and AC recording of electrical activity. The figure shows the inhibitory effect on electrical and mechanical activity of stepwise increases in $[Mg^{2+}]_o$ from 1.2 (A) to 2.4 (B), 4.8 (C) and 10 mM (D)

Under conditions where no spikes can appear the effect of raising $[Mg^{++}]_o$ on the amplitude of K^+-contractures was studied. Fig. 1 shows that the sustained contracture amplitude was unaltered when $[Mg^{++}]_o$ was increased from 0 to 10 mM in K-tris with 2.5 mM Ca. The first, phasic part of the K^+-response decreased like the spontaneous activity as the $[Mg^{++}]_o$ was increased. A quantitation of the Mg^{++} effect on the K^+-contractures in different $[Ca^{++}]_o$ is shown by the broken lines in Fig. 4. The amplitude of the sustained K^+-contractures is not altered by increase in $[Mg^{++}]_o$ for extracellular Ca^{++}-concentrations between 0.5 and 10 mM, whereas for $[Ca^{++}]_o < 0.5$ mM a decrease in response is seen for the higher $[Mg^{++}]_o$. The smaller the $[Ca^{++}]_o$, the more sensitive does the muscle become to changes in $[Mg^{++}]_o$.

As the solution with 0 mM Mg^{++} was only nominally Mg-free it was of interest to decrease $[Mg^{++}]_o$ further. This was done by adding 1 mM EDTA to the nominally Mg^{++}–Ca^{++}-free Na-tris. EDTA forms complexes with divalent cations. According to Hubbard *et al.* (1968) the concentration of free Mg^{++} and Ca^{++} in this solution is below $10^{-[illegible]}$ and $10^{-[illegible]}$ M respectively. When placed in this nominally Mg^{++}–Ca^{++}-free solution with 1 mM EDTA the muscle

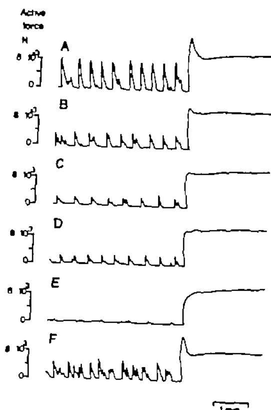


Fig. 1 In the left half is shown the effect on the spontaneous activity of one preparation by increasing the $[Mg^{++}]_o$ stepwise from 0 (A) to 1.2 (B), 2.4 (C), 4.8 (D) and 10 mM (E). In (F) is shown how the muscle recovers in solution with 0 mM Mg^{++}. In the right half are shown potassium contractures in the respective solutions.

corresponding decrease in the number of spikes is demonstrated when $[Mg^{++}]_o$ is raised. To see if this membrane effect was due to hyperpolarization the concentration of K^+ in Na-tris 2.5 mM Ca^{++} 10 mM Mg^{++} was increased from 6 to 12 mM. The spontaneous electrical and mechanical activity then became normalized *i.e.* as in Na tris 2.5 mM Ca 1.2 mM Mg^{++} solution (not shown in the figure).

The electrical activity seen in Fig. 2 shows the mass activity in the whole muscle. From this kind of registration it is not possible to determine whether all parts of the preparation participate in the phasic contractions. Therefore one cannot exclude the possibility that besides the diminished number of spikes per burst, there may be blocking of propagation when $[Mg^{++}]_o$ is raised. By using three extracellular glass capillary electrodes placed on different parts of the preparation, it was possible to obtain information on how the electrical activity propagated along the muscle. Fig. 3 A shows that the areas under the three electrodes were all activated and showed similar burst durations during a contraction when $[Mg^{++}]_o$ was 1.2 mM. When $[Mg^{++}]_o$ was increased the propagation was impaired. First in solution with 5 mM Mg^{++} (B) the number of spikes in each burst decreased as it propagated along the preparation and finally in solution with 10 mM Mg^{++} some of the bursts failed to propagate to the electrode most remote from the pacemaker area (C).

A quantitation of the Mg^{++} effect on the spontaneous activity in different $[Ca^{++}]_o$ is shown by the full lines in Fig. 4. The integrated spontaneous mechanical activity decreased rapidly when $[Mg^{++}]_o$ was increased for all $[Ca^{++}]_o$ studied.

In order to see if Mg^{++} would influence the degree of activation of the contractile apparatus

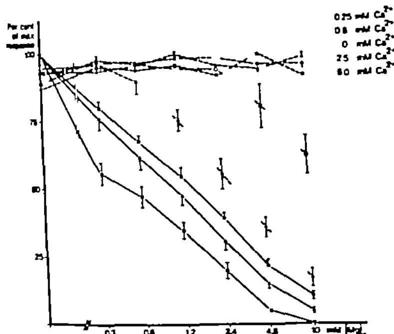


Fig. 4 Quantitative effects of stepwise increase in $[Mg^{++}]_o$ from 0–10 mM in solutions with various $[Ca^{++}]_o$ expressed as percentages of the maximal force. Broken lines show effects on potassium contractures, full lines effects on integrated spontaneous activity. Each point represents the mean of five or more measurements. Vertical bars represent S.E.

1.2 mM Mg^{++} and became spontaneous active again. The other (lower recording) was placed in Na-tris 2.5 mM Ca^{++} but 0 mM Mg^{+} as before and nothing happened (E). In F the muscles were depolarized in K-tris 2.5 mM Ca^{++} 1.2 mM Mg^{++} (upper recording) and K-tris 2.5 mM Ca^{++} 0 mM Mg^{++} (lower recording) after a 5 min period in Na-tris 0 mM Ca^{++} 1.2 mM Mg^{++} (upper recording) and Na-tris 0 mM Ca^{++} 0 mM Mg^{++} (lower recording). The muscle which had had Mg^{+} (upper recording) developed a relatively strong contracture, but the Mg^{++}-depleted muscle (lower recording) gave only a very small response. In G is shown how both muscles recovered in Na-tris 2.5 mM Ca^{++} 1.2 mM Mg^{++}

Discussion

In 1968 Viveros and Somjen found that resistance vessels in the gracilis muscle of the dog in vivo dilate when exposed to a high concentration of Mg^{++} in the blood. They thought that this dilatation was caused by a direct action of free Mg^{+} on the smooth muscle cells of the vessels by preventing the entry of Ca^{++} from the extracellular fluid into the muscle cell or in some other way interfering with excitation–contraction coupling. These results were confirmed in vitro by Altura and Altura (1974) and Jurevics and Carrier (1973). Our results show that increased $[Mg^{++}]_o$ has an inhibitory effect on the spontaneous electrical and mechanical activity of the rat portal vein. Maximal integrated force was obtained in Mg-

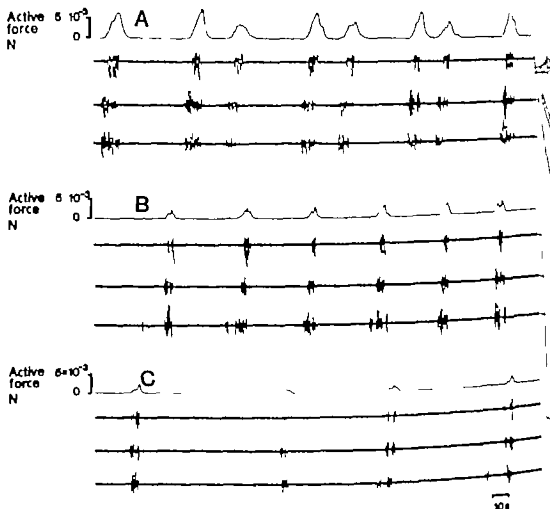


Fig. 3. Recording of mechanical activity and electrical activity from 3 electrodes placed on different sites on the muscle. The muscle in solutions with 1.2 mM Mg^{2+} (A), 4.8 mM (B), and 10 mM (C). With increasing $[Mg^{2+}]_o$ there is a decrease in spike activity and in C impaired spike propagation is seen.

lost spontaneous activity as would be expected merely from the low $[Ca^{2+}]_o$. After 1 h in this medium, the muscles were placed in Na tris with 2.5 Ca but with no Mg^{2+} for 1/2 h. No activity appeared. When Mg^{2+} was added the muscle again became spontaneously active. The result of one such experiment is shown in Fig. 5. Recordings were obtained from 2 preparations (upper and lower tracings). In A the muscles were spontaneously active in Na-tris solution with 2.5 mM Ca and 1.2 mM Mg. The muscles were then placed in Na tris 0 mM Ca 1.2 mM Mg^{2+} for 5 min and then in K-tris with 2.5 mM Ca^{2+} 1.2 mM Mg^{2+} (arrow). Contractures developed (B). After this the muscles relaxed in Na-tris 0 mM Ca 1.2 mM Mg^{2+} for a few min and they were then transferred to nominally Ca Mg^{2+} free Na tris with 1 mM EDTA (C). One hour later the solution was changed to Na-tris 2.5 mM Ca 0 mM Mg^{2+}. No spontaneous activity appeared and only a very small contracture was seen when the muscles were depolarized (arrow) in K-tris 2.5 mM Ca 0 mM Mg (D). One of the muscles (upper recording) was now placed in Na tris 2.5 mM Ca

...ects the excitation-contraction coupling (Altura and Altura 1974, Jurevics and ... 1973)

...phy *et al.* (1969) compared the Mg^{+} dependence of skeletal and arterial smooth ... actomyosin. They found that for skeletal muscle the response of the contractile ... reached maximum at about 10^{-4} M Mg^{+} when the Ca and ATP concentrations ... optimal. They did not succeed in defining precisely the Mg^{+} dependence for arterial ...yosin but found the highest rates of ATP-ase activity when the free-Mg concentration ...timated 10^{-4} M.

...ty (1971, 1974) has found on the rat tail artery that only half of the tissue Mg is lost ... the smooth muscle is incubated in Mg-free Krebs for 24 h. In this Mg-free solution ... was first a rapid loss of tissue Mg depending on the original $[Mg^{+}]_{e}$ followed by a slow ...onent related to the content of intracellular compounds forming complexes with Mg^{+} ... Mg^{++} is lost in Mg-Ca-free solution because external Ca markedly decreases the ...eability of the cell membrane to passive movements of Mg^{+}. Palaty also found in ...bolically active muscle that the $[Mg^{+}]_{i}$ is maintained at a level of about 10^{-4} M. This ...ch lower than in his normal Krebs solution indicating that the muscle appears to ... an active Mg^{+} extrusion mechanism.

... our experiments the portal vein could maintain its increased contractile activity in ...free solution for hours. When also Ca^{++} was removed from this solution and 1 mM ...TA was added, the muscle relaxed. 1 mM EDTA in nominally Mg-Ca-free solution ...ers the free $[Mg^{+}]$ below 10^{-6} M and free [Ca] below 10^{-9} M (Hubbard *et al.* 1968). ...en the muscle after 1 h in this solution was placed in the Mg-free Na-tris with 2.5 mM ... it remained inactive. Contractile activity reappeared when 1.2 mM Mg was added. It ...ms that during the period in Mg-Ca-free solution with 1 mM EDTA cell Mg is lowered ...neath the Mg^{+} concentration which is needed for spontaneous activity. It is shown in ...5 that a depolarization of the muscle by K-tris 2.5 Ca 0 Mg^{++} gave only a contracture ...about 10% of that obtained when 1.2 mM Mg^{+} had also been added to the solution.

...present study was supported by grants from the Swedish Medical Research Council (20-14 X), from ... Medical Faculty, University of Lund, and from AB Hässle, Göteborg, Sweden. Thanks are due to Mrs ...Lundahl, Mrs Monica Hedenholm and Miss Ina Gunnarsson for able technical assistance.

References

...ALTURA, B. M. and B. T. ALTURA, Magnesium and contraction of arterial smooth muscle. *Microvasc. Res.* 1974. 7. 145–155.

...JOHANSSON, J., B. WAHLSTRÖM, B. JOHANSSON and O. JONSSON, Influence of the ionic environment on spontaneous electrical and mechanical activity of the rat portal vein. *Circulat. Res.* 1967. 21. 609–618.

HUBBARD, J. I., S. F. JONES and E. M. LANDAU, On the mechanism by which calcium and magnesium affect the spontaneous release of transmitter from mammalian motor nerve terminals. *J. Physiol.* (Lond.) 1968. 196. 355–380.

JOHANSSON, B. and S. MELLANDER, Static and dynamic components in the vascular myogenic response to passive changes in length as revealed by electrical and mechanical recordings from the rat portal vein. *Circulat. Res.* 1975. 36. 76–83.

...HART, H. A. and O. CARRIER, JR., Effects of magnesium on responses of aortas from normal and reserpine treated rabbits. *Amer. J. Physiol.* 1973. 225. 1479–1485.

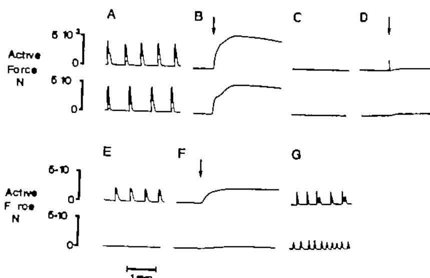


Fig. 5 The effect of a Mg-Ca-free solution with 1 mM EDTA on the spontaneous activity and potassi co tractures in two preparations of the rat portal vei For details see text.

free solution, and a stepwise increase in [Mg]ₒ decreased the mechanical and electri activity (Fig. 1) There was a decrease in contraction amplitude and in some preparations tendency to a decrease in the frequency of contractions At 10 mM [Mg]ₒ the preparatio usually had no spontaneous activity at all

In the sucrose-gap expts it was clearly shown that increasing [Mg]ₒ within the low concentration range led to comparable inhibition of electrical and mechanical activity the highest Mg concentration (10 mM) the spikes had become so small that the spike cou ter underestimated their number The decrease in mechanical activity is most likely due inhibition of the electrical activity A possible membrane effect of Mg could be an i pairment of conductance between the cells. Our results with 3 glass capillary electrod showed that for the higher Mg concentrations the spike propagation was impaired (Fi 3 C) The results described above can be explained largely as an hyperpolarizing effect Mg^{++} as a moderate increase in [K]ₒ (12 mM) eliminated the inhibitory action of Mg

It was considered possible that Mg besides the hyperpolarizing action on the spo taneously active muscle might influence the degree of activation of the potassium depolariz muscle. It was found (Fig. 1 and 4) that Mg in concentrations between 0 and 10 mM d not influence the steady amplitude of the potassium induced contraction when [Ca]ₒ wa between 0.5 and 5 mM Only when [Ca]ₒ was 0 5 mM did increases in [Mg]ₒ affe the contraction amplitude. It is probable that Mg can affect Ca permeability fo [Ca]ₒ < 0 5 mM For higher [Ca]ₒ intracellular Ca concentration might become larg than that needed for complete activation of the contractile apparatus. A moderate decreas of Ca permeability would therefore not have any effect on the contractile output.

It was noted that the initial fast phase of the K response which is thought to be the resul of liberation of superficially bound Ca (Sigurdsson, Uvelius and Johansson 1975) becam less prominent when the [Mg]ₒ was increased It is likely that Mg affects the spike appearing during the fast phase (Sigurdsson, Uvelius and Johansson 1975) or in some othe

y affects the excitation-contraction coupling (Altura and Altura 1974, Jurevics and ...rner 1973).

Murphy *et al.* (1969) compared the Mg dependence of skeletal and arterial smooth muscle actomyosin. They found that for skeletal muscle the response of the contractile system reached maximum at about 10^{-4} M Mg when the Ca and ATP concentrations were optimal. They did not succeed in defining precisely the Mg dependence for arterial actomyosin but found the highest rates of ATP-ase activity when the free-Mg concentration approximated 10^{-4} M.

Palaty (1971, 1974) has found on the rat tail artery that only half of the tissue Mg is lost when the smooth muscle is incubated in Mg-free Krebs for 24 h. In this Mg-free solution there is first a rapid loss of tissue Mg depending on the original [Mg]$_o$ followed by a slow component related to the content of intracellular compounds forming complexes with Mg. Less Mg^{++} is lost in Mg–Ca-free solution because external Ca markedly decreases the permeability of the cell membrane to passive movements of Mg. Palaty also found in metabolically active muscle that the [Mg]$_i$ is maintained at a level of about 10^{-4} M. This is much lower than in his normal Krebs solution indicating that the muscle appears to possess an active Mg^{++} extrusion mechanism.

In our experiments the portal vein could maintain its increased contractile activity in Mg-free solution for hours. When also Ca was removed from this solution and 1 mM EDTA was added, the muscle relaxed. 1 mM EDTA in nominally Mg–Ca-free solution lowers the free [Mg^{++}] below 10^{-4} M and free [Ca] below 10^{-9} M (Hubbard *et al.* 1968). When the muscle after 1 h in this solution was placed in the Mg-free Na-tris with 2.5 mM Ca^{++} it remained inactive. Contractile activity reappeared when 1.2 mM Mg was added. It seems that during the period in Mg–Ca-free solution with 1 mM EDTA cell Mg is lowered beneath the Mg^{++} concentration which is needed for spontaneous activity. It is shown in Fig. 5 that a depolarization of the muscle by K-tris 2.5 Ca 0 Mg gave only a contracture of about 10^{-} of that obtained when 1.2 mM Mg^{++} had also been added to the solution.

The present study was supported by grants from the Swedish Medical Research Council (28-14 X), from the Medical Faculty, University of Lund, and from AB Hässle, Göteborg, Sweden. Thanks are due to Mrs Kerstin Lundahl, Mrs Monica Heidenholm and Miss Ina Gonnarsson for able technical assistance.

References

ALTURA, B. M. and B. T. ALTURA, Magnesium and contraction of arterial smooth muscle. *Microvasc. Res.* 1974. 7. 145–155.

AXELSSON, J., B. WAHLSTRÖM, B. JOHANSSON and O. JONSSON, Influence of the ionic environment on spontaneous electrical and mechanical activity of the rat portal vein. *Circulat. Res.* 1967. 21. 609–618.

HUBBARD, J. I., S. F. JONES and E. M. LANDAU, On the mechanism by which calcium and magnesium affect the spontaneous release of transmitter from mammalian motor nerve terminals. *J. Physiol.* (Lond.) 1968. 194. 355–380.

JOHANSSON, B. and S. MELLANDER, Static and dynamic components in the vascular myogenic response to passive changes in length as revealed by electrical and mechanical recordings from the rat portal vein. *Circulat. Res.* 1975. 36. 76–83.

JUREVICS, H. A. and O. CARRIER, JR. Effects of magnesium on responses of aortas from normal and reserpine treated rabbits. *Amer. J. Physiol.* 1973. 225. 1479–1485.

LIVINGSTONE, D. M. and W. E. C. WACKER, The role of magnesium in neuromuscular physiology. Triangle 1971 10 169–171

LJUNG, B. and L. STAGE, Adrenergic excitatory influences on initiation and conduction of electrical activity in the rat portal vein. *Acta physiol. scand.* 1970, 80 131–141

MURPHY, R. A., D. F. BOHR and D. L. NEWMAN, Arterial actomyosin: Mg, Ca and ATP ion dependence for ATPase activity. *Amer. J. Physiol.* 1969 217 666–673.

PALATY, V. Distribution of magnesium in the arterial wall. *J. Physiol.* (Lond). 1971 353–368

PALATY, V., Regulation of the cell magnesium in vascular smooth muscle. *J. Physiol.* (Lond.) 1974 X 555–569

SIGURDSSON, S. B. and B. UVELIUS, Influence of magnesium on electrical and mechanical activity of the isolated rat portal vein. *Blood Vessels* 1975 12 376.

SIGURDSSON, S. B., B. UVELIUS and B. JOHANSSON, Relative contribution of superficially bound and extracellular calcium to activation of contraction in isolated rat portal vein. *Acta physiol. scand.* 1975 [illegible] 263–269

VIVEROS, H. and G. G. SOMJEN, Magnesium–calcium antagonism in the contraction of arterioles. *Experientia* (Basel) 1968, 24 457–459

WALSER, M. Magnesium metabolism. *Ergebn. Physiol.* 1967 59 185–235

Acta physiol. scand. 1977 99 377–384
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Quantitative Evaluation of the Blood–Brain Barrier Capacity to Form Dopamine from Circulating L-DOPA

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Abstract

Hardebo, J. E., L. Edvinsson, Ch. Owman and E. Rosengren. *Quantitative evaluation of the blood–brain barrier capacity to form dopamine from circulating L-DOPA*. Acta physiol. scand. 1977 99 377–384

The ability of the blood–brain barrier to form dopamine from increasing doses of systemically administered L-DOPA has been studied in rats by a combination of chemical determination of dopamine, and histochemical and cytofluorometric measurements of L-DOPA and dopamine. The break-through of L-DOPA from the circulation into the brain parenchyma via the enzymatic blood–brain barrier was estimated by comparing the amount of newly formed dopamine in the caudate nucleus-putamen and in the cerebellum. The capillaries were found to efficiently trap L-DOPA in their walls, and an upper limit was reached (at an administered i.p. dose of 100 mg/kg of L-DOPA). It could be estimated that approximately 3 % of the total dose of L-DOPA given was decarboxylated by the blood–brain barrier. The possible influences by the regional differences in perfusion of the two regions seen after administration of L-DOPA was ruled out in measurements of local cerebral blood flow using the ^{14}C-ethanol technique.

The capillaries in the central nervous system are capable of decarboxylating certain amine precursors, such as dihydroxyphenylalanine (DOPA) (Bertler *et al.* 1966, Owman and Rosengren 1967 Bartholini *et al.* 1971), 5-hydroxytryptophan (Bertler Falck and Rosengren 1964), and dihydroxyphenylserine (Constantinidis *et al.* 1975), thus impeding the passage of these amino acids into the brain. This blood–brain barrier property appears to be a general feature among several laboratory animals studied, as well as in humans (Hardebo *et al.* 1976). In peripheral tissues these amine precursors pass freely through the vessel walls into the surrounding tissues, probably because the vessel wall is devoid of a decarboxylating capacity (Owman and Rosengren 1967). The enzymatic barrier mechanism is interesting both from a general biological standpoint, constituting one special type of blood–brain barrier function (see Rapoport 1976), and clinically in the medical therapy of Parkinson disease (Cotzias, Papavasiliou and Gellene 1969).

LIVINGSTONE, D M. and W E. C. WACKER, The role of magnesium in neuromuscular physiology. *T* [illegible]
1971 *10.* 169–171
LJUNG, B. and L. STAGE, Adrenergic excitatory influences on initiation and conduction of electrical activity in the rat portal vein. *Acta physiol scand* 1970, *80* 131 141
MURPHY R. A., D F BOHR and D L. NEWMAN, Arterial actomyosin Mg, Ca and ATP ion dependence for ATPase activity *Amer J Physiol* 1969 *217* 666–673
PALATY V Distribution of magnesium in the arterial wall. *J Physiol.* (Lond) 1971 353–368.
PALATY V Regulation of the cell magnesium in vascular smooth muscle. *J Physiol.* (Lond) 1974 [illegible]
555–569
SIGURDSSON, S. B and B. UVELIUS, Influence of magnesium on electrical and mechanical activity of the isolated rat portal vein. *Blood Vessels* 1975 *12.* 376.
SIGURDSSON S B., B UVELIUS and B. JOHANSSON Relative contribution of superficially bound and extracellular calcium to activation of contraction in isolated rat portal vein. *Acta physiol. scand*
263–269
VIVEROS, H and G G SOMLYO Magnesium–calcium antagonism in the contraction of arterioles. *I*
(Basel) 1968. *24* 457–459
WALSER, M Magnesium metabolism *Ergebn. Physiol.* 1967 *59* 185–235

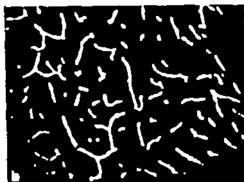


Fig. 1. Animal pretreated with 5 mg/kg of reserpine and 100 mg/kg of nialamide. 20 min after the injection of 100 mg/kg of L-DOPA, an intense green fluorescence develops in the brain capillary walls. Note the similar density of the capillary network in the caudate nucleus (a) and the cerebellum (b). The parenchymal "background" in both regions is essentially non-fluorescent. Magnification, 90

Results

Fluorescence microscopy of the caudate nucleus from non-treated control animals showed an intense, green fluorescence in the parenchyma deriving from the very dense network of delicate dopamine-containing axon terminals (*cf.* Fuxe 1965, Lindvall and Björklund 1974). The parenchyma in the cerebellum, on the other hand, was essentially non-fluorescent and only a small number of isolated green-fluorescent noradrenaline axons was present in the preparations. The walls of the parenchymal blood vessels in both regions were non-fluorescent, except for the presence of the perivascular sympathetic nerve fibres (*cf.* Owman *et al.* 1974).

Reserpine treatment was used with the aim of depleting the dopamine from the caudate nucleus to make it better comparable with the cerebellum—which contains no measurable dopamine (Bertler *et al.* 1966)—in terms of the basal catecholamine level. A dose of 5 mg/kg (chosen from a series of pilot expts.) reduced the concentration of dopamine after 4 h to a mean of 0.45 ± 0.15 μg/g, which is approximately 5% of the normal dopamine level.

Administration of 60 mg/kg of L-DOPA to reserpinized animals, in which monoamine oxidase had been inhibited by nialamide, resulted in a green fluorescence of equally high intensity in the capillary walls of the two regions analyzed, whereas the parenchyma remained essentially non-fluorescent (Fig. 1). It has previously been shown that the capillary fluorescence reflects the local formation of dopamine from the exogenous precursor amino acid (Bertler *et al.* 1966).

The extent of dopamine formation in the cerebellar and striatal tissues was followed after administration of increasing doses of L-DOPA (Fig. 2). It can be seen that a similar amount was formed in both regions up to a dose level of 100 mg/kg of L-DOPA, after which the concentration in the cerebellum levelled out, despite the administration of increasing doses of precursor. In the caudate nucleus-putamen the increment in the dopamine formation continued, so that a markedly and significantly higher concentration was achieved upon 140 to 200 mg/kg of L-DOPA as compared with cerebellum (Fig. 2).

It can be assumed that the decarboxylation capacity in the brain capillary wall has a lim which means that the amino acid, for example L DOPA, penetrates into the brain pare chyma at particularly high circulating concentrations. In regions, such as cerebellum, taining no dopamine-storing neurons (Lindvall and Björklund 1974), where the forma of measurable amounts of dopamine thus can be expected to take place only in the capil walls, the concentration of dopamine will come to a maximum when the limit is reache However in other areas, such as the caudate nucleus, the dopamine concentration w continue to increase beyond this limit namely when the large amount of dopamin nerves in this region (Fuxe 1965 Lindvall and Björklund 1974) becomes exposed to t L DOPA passing into the brain parenchyma. This hypothesis was tested in the present stu by administration of increasing doses of L DOPA to rats, followed by chemical determin tions of the amount of newly formed dopamine in the caudate nucleus and cerebellum.

Material and Methods

Animals. The study was performed on female Sprague-Dawley rats weighing 190–200 g and fed ith sta ard pellets and tap water *ad lib* One group of 10 non-treated animals served as controls. The remain (100 a imals) were pretreated with 5 mg/kg i.p. reserpine (Serpasil, Ciba) followed 4 h later by the mo am ne o idase inhibit r nialamide (Niamid, Pfizer 100 mg/kg i.p). One hour later 88 animals were jected with increasi g doses (20 40 60 80, 100, 140 200 and 300 mg/kg i p.) of L DOPA (L 3,4-dihydro phenylalanine, Sigma) fully dissolved in 0 9% saline, nd killed 20 min later

Fluorescence microscop Immediately after decapitation, small tissue pieces were dissected out from cerebellar hemisphere nd caudate ucleus-putamen on the right side frozen to the temperature of liq nitrogen and further processed for fluorescence monoamine histochemistry according to Björklund Fa and Owman (1972). The paraformaldehyde used in the histochemical procedure has previously been e librated with ir of 70 humidity The formaldehyde-induced, histochemically visible fluorophore both catecholamines and DOPA have the same spectral characteristics, with excitation/emission max at 410/470–480 mμ (Björklund *et al* 1972), and exh bit a green light under the optical conditions used

The cerebrovascular system was outlined some of the nimals by perfusion with India ink (Ekl 1966) through the left ventricle of the heart under ether nesthesia

Cytofluorometry Specimens processed f fluorescence histochem stry s above ere used. The cereb a d striatal tissues were embedded side-by-side in the same paraffi block and sectioned simultaneo at 5 μm thickness. The intensity of the f rmaldehyde-induced fluorescence in the capillary ll and in adjacent parenchyma was compared, using Leitz microspectrograph (Björklund Ehinger and Falck 1 at ×5 bjective magnification. A mercury lamp (Osram HBO 200 W/2) was used as light source, monochromator at the excitation sid was fixed t 405 m, a d filter with its edge t 430 nm was use barrier The entrance slit of the monochromator t the emission side was pen between 460 and 480 The photomultiplier ignals expressing relative fluorescence were read on galvanometer The procedure of locating the fluorescent structure focusing, and obtai ing the measurement response less than 30 s, during which time the UV ph todecomposition by the light source has been show to am only about 5% of the initial fluorescence tensity (Jonsson 1971).

Chemical determinations After killing of the nimals, the entire cerebell m and the caudate nucl putamen tissue of both sides were dissected out and homogenized separately in ice-cold 0 4 N perchl acid for fluorometric determination of dopamine according t A ton and Sayre (1964). One rat as for each determination.

Blood flow measurements. Cerebral blood flow wa measured by radioactive clearance technique cording t the procedure described by Eklöf *et al.* (1974), using ethanol-C[1] as indicator The scintilla countings were carried out on cerebellar preparation weighing appro imately 25 mg and consisting both grey and white matter and striatal preparation (bout 25 mg) consisti g of caudate nucleus-puta tissue from both sides.

Statistics. Mean values were compared accordi g to Student test. A Hewlett Packard desk comp was used in the calculations.

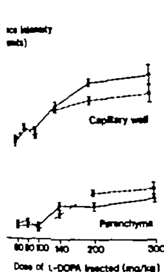


Fig. 3

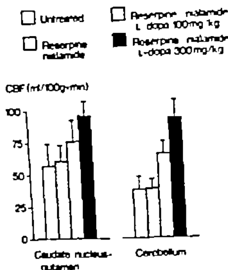


Fig. 4

Fig. 3 Cytofluorometric measurement of formaldehyde-induced fluorescence (of DOPA and dopamine) capillary wall and parenchyma from caudate nucleus-putamen (● — ●) and cerebellum (O— O) of rats ... the same treatment as described in Fig. 2. Mean ± SEM of 8 measurements at each dose level.

Fig. 4 Regional cerebral blood flow (CBF) in caudate nucleus-putamen and cerebellum of untreated ani... and after administration of two dose-levels of L-DOPA to animals pretreated with reserpine and nialamide. Mean ± SEM, 5 determinations in each group.

... an equally low endogenous base level of the amine in the two structures. Therefore, reserpine was given in a series of pilot expts. to find out the lowest dose that, during the shortest time of treatment, would cause sufficient reduction in the high dopamine content of the striatal tissue. Although a dose of 5 mg/kg after 20 h resulted in a complete depletion of striatal dopamine, a shorter time of 4 h (reducing the dopamine level by approximately ...) was chosen because the animals were considered to be in too bad general condition after the long-term reserpinization. Subsequent treatment with a monoamine oxidase inhibitor (nialamide) was given to avoid break-down of the amine formed from the exogenous L-DOPA, in the brain parenchyma as well as in the capillary walls (Bertler *et al.* 1966).

In view of the working hypothesis mentioned in the introduction, the results would indicate that the upper limit of entrapment capacity for L DOPA in brain capillaries is reached (at an intraperitoneal dose level of 100 mg/kg given 20 min before the animal was killed). At higher doses, part of the amine precursor appears to leak beyond the capillary wall into the brain parenchyma: the increment in the concentration of dopamine in the caudate nucleus-putamen continues (Fig. 2), reflecting that dopamine is now being formed not only in the capillary wall but also in the parenchyma of this tissue. In the cerebellum there was no corresponding increase after the upper limit of entrapment capacity had been reached, the tendency to a slight increment in dopamine formation here at the highest L DOPA dose may be due to the appearance of measurable amounts of dopamine in the (non-dopaminergic)

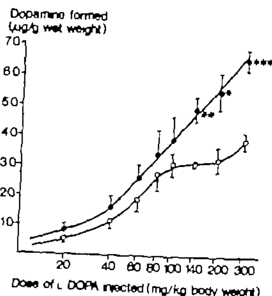


Fig. 2. Semilogarithmic representation of dopa[mine] formed (expressed as µg per gram wet weig[ht]) caudate nucleus-putamen (●—●) and cereb[ellum] (O—O) of rats pretreated with reserpine (5 m[g/kg]) 4 hrs before L DOPA in increasing i.p. doses; an[imals] killed 20 min later. Mean ± SEM. 6 fluoro[metric] determinations at each dose level. Dopam[ine in] animals receiving no L DOPA, caudate nuc[leus-] putamen, 0.45 ± 0.15 µg/g; cerebellum, non-m[eas]urable level. Comparison of matching mean v[alues] f[or] the two tissues by Student's t-test: *p ... *0.001 < p < 0.01; other differences non-signif[icant].

In the fluorescence microscope a capillary fluorescence could be detected already at lowest L DOPA dose (20 mg/kg) though the intensity of the emitted light was only we[ak]. It became higher with increasing doses of exogenous precursor until 100–140 mg/kg a[fter] which no further increment could be seen. The "background" fluorescence in the par[en]chyma was low up to the same dose level of L DOPA after which a clear increase, similar [in] the two regions, could be detected at the two highest doses of L DOPA.

The density of the capillary network was compared in the cerebellar and striatal tissues [on] the basis of the arrangement of fluorescent capillary walls as well as the appearance of In[dia] ink loaded microvessels. No difference was found in either white or grey matter in the t[wo] regions (Fig. 1).

In order to obtain a more quantitative estimation of the histofluorescence changes in t[he] capillary wall and parenchyma, intensity measurements were carried out on sections fro[m] the two regions. It can be seen from Fig. 3 that the capillary wall fluorescence was alwa[ys] significantly higher than the corresponding parenchymal fluorescence. Further in t[he] cerebellar and striatal tissues the pattern of increase in fluorescence with increasing doses [of] L DOPA was similar in capillaries and parenchyma, respectively.

The blood flow in the caudate nucleus-putamen and cerebellum was measured in un[treated] controls and after the drug injections. The flow under normal conditions (Fig. 4) was lower in the cerebellum, though the difference was not statistically significant. Combine[d] treatment with reserpine followed by nialamide had no effect on flow in either region. How[ever] when L DOPA was added to the treatment an increased blood flow could be measured; this increment was more pronounced in the cerebellar tissue.

Discussion

In order to allow for comparison of the dopamine formation in the preparation of caudate nucleus-putamen and the cerebellar tissue after the L DOPA injections, it was desirable to

of L-DOPA used in the present study it could be expected that a plateau in dopamine accumulation should form not only in the cerebellum but also in the striatal tissue.

Attempts have previously been made to determine the capacity of the enzymatic barrier not only on the basis of semiquantitative estimation of fluorescence intensities using the formaldehyde method (Bertler et al. 1966, Dowson and Laszlo 1971 de la Torre and Mullan 1970). Varying results have been reported, apparently because the approaches used have been less precise than the combination of techniques applied in the present experimental model.

The efficiency of the blood–brain barrier to impede the passage of L DOPA into the brain by a decarboxylating mechanism, in addition to the decarboxylation activity in the peripheral tissues, have required high doses of L DOPA in the medical treatment of Parkinson's disease with disturbing side-effects as a frequent result. Partly based on the knowledge about the functions of this special type of enzymatic barrier (see Owman et al 1974 Hardebo et al 1976) it has now been possible to overcome, to a considerable extent, these problems: a decarboxylase inhibitor (Ro 4-4602 or MK 486), effective in peripheral tissues as well as in brain capillary walls but only to a negligible extent in the brain parenchyma, is added to L-DOPA in the therapy. Under these conditions, the amine precursor can be administered in lower doses, reducing the incidence of undesired side-effects (Mars 1973 Miller and Wiener 1974).

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References

Anton, A. H. and D. F. Sayre, The distribution of dopamine and dopa in various animals and a method for their determination in diverse biological material. *J. Pharmacol. exp. Ther.* 1964, *145* 326–336.

Bartholini, G., J. Constantinidis, R. Tissot and A. Pletscher, Formation of monoamines from various amino acids in the brain after inhibition of extracerebral decarboxylase. *Biochem. Pharmacol.* 1971 *20* 1243–1247.

Bertler, Å., B. Falck, Ch. Owman and E. Rosengren, The localization of monoaminergic blood–brain barrier mechanisms. *Pharmacol. Rev.* 1966, *18* 369–385.

Bertler, Å., B. Falck and E. Rosengren, The direct demonstration of a barrier mechanism in the brain capillaries. *Acta pharmacol.* (Kbh.) 1964, *20* 317–321.

Björklund, A., B. Ehinger and B. Falck, A method for differentiating dopamine from noradrenaline in tissue sections by microspectrofluorometry. *J. Histochem. Cytochem.* 1968, *16* 263–270.

Björklund, A., B. Falck and Ch. Owman, Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic amines. In: S. A. Berson (ed.) *Methods of Investigative and Diagnostic Endocrinology*. *Vol. 1*. J. E. Rall and I. J. Kopin (eds.) *The Thyroid and Biogenic Amines*. North Holland, Amsterdam. 1972, p. 318–368.

Cotzias, G. C., P. S. Papavasiliou and R. Gellene, Modification of parkinsonism: Chronic treatment with L-dopa. *New Engl. J. Med.* 1969, *280* 337–345.

Constantinidis, J., F. Geissbuhler, J. M. Gaillard, C. Aubert, Th. Hovaguimian and R. Tissot, Formation de noradrénaline dans le cerveau du rat après administration de (–)-erythro-3,4-dihydroxyphénylsérine. *Psychopharmacologia* (Berl.) 1975 *41* 201–209.

Dowson, J. H. and I. Laszlo, Quantitative histochemical studies of formaldehyde-induced parenchymal fluorescence following L-dopa administration. *J. Neurochem.* 1971 *18* 2501–2508.

Ehinger, B., Connections between adrenergic nerves and other tissue components in the eye. *Acta physiol. scand.* 1966, *67* 57–64.

monoamine nerves exposed to the L DOPA leaking into the cerebellar parenchyma. On t
basis of the maximum amount of dopamine formed in the cerebellum (3 12 μg/g) occurs
at an i p dose of 100 mg/kg of L DOPA it can be estimated that approximately 3 % of t
exogenous amino acid is decarboxylated in the blood brain barrier (whereas the remaind
of the conversion takes place in peripheral tissues).

There is evidence already at the level of fluorescence microscopy both in the present a
previous (Bertler *et al* 1966) studies, that the fluorophores which can be visualized aft
administration of L DOPA at low doses are located only in the microvessel walls, wher
at high doses of L DOPA an increasing intensity of fluorescence becomes visible also in t
parenchyma, where it has a diffuse localization A similar diffuse parenchymal fluoresce
starts to appear also at low L DOPA doses following inhibition of the decarboxylase (in t
capillary walls) (Bertler *et al* 1966) In spite of these fluorescence microscopic findings
would still be possible that at least part of the steady increase in striatal dopamine formati
measured chemically (Fig. 2) would be explainable by a higher decarboxylase activity in
capillary walls of the striatal compared with the cerebellar vascular beds. However this i
contradicted by the cytofluorometric measurements showing that the pattern of increa
fluorescence intensity was very similar in the capillary walls from the two regions (Fig
Also the studies by Wade and Katzman (1975) support the view that the decarboxyl
activity at the level of the capillary wall is similar in various brain regions (having eq
brain uptake indices for L DOPA after a single capillary circulation). The intensity of
capillary fluorescence continued to increase even when the limit of capillary decarboxyl
capacity had been reached. This can be explained by the continued capillary uptake
DOPA whose fluorescence is added to the dopamine fluorescence in the capillary en
thelium The cytofluorometric measurements of the intensity of the diffuse fluorescenc
the parenchyma agreed with the fluorescence microscopic observations in that a dist
increase in this fluorescence did not occur until a dose of above 100 mg/kg of L DO
(*i.e* when the limit of capillary decarboxylation capacity had been reached). The differen
between the curves for the fluorometric determinations (Fig. 2) and the cytofluorometr
measurements (Fig. 3) thus lies in the fact that the former shows dopamine specifically
simultaneously in the microvasculature walls and the parenchyma whereas the latter wl
measures the fluorescence selectively in the microvasculature walls and parenchyma, can
discriminate between the fluorophores of DOPA and dopamine (Björklund *et al* 1972)

The fluorescence microscopic analysis indicated that the density of the capillary netw
was equal in the two regions studied The regional measurements of cerebral blood f
moreover revealed that the increase in flow upon L DOPA injection was more pronoun
in the cerebellum. This shows that the significantly higher dopamine formation in the stri
tissue at L DOPA doses above 100 mg/kg could not simply be due to a better perfusio
this region compared with the cerebellum.

In isolated perfused rat brains, Horst *et al* (1974) have found that the dopamine accum
tion in the tissue during perfusion with increasing concentrations of L DOPA does
proceed in a continuous elevation, but progresses through a series of plateaus and regressi
This has been interpreted as some kind of "autoinhibition" of L DOPA uptake an
dopamine formation (Horst *et al* 1974). If such a mechanism was involved at the dose le

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The Compliance Curve for the Flow Limiting Segments of the Airway I Model Studies

By

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Abstract

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By means of [illegible] post-stasis tube airway compliance curves describing the cross-sectional area (A) as function of transmural pressure (Ptm) were constructed for several locations in the elastic airway of a mechanical model of the lung. From these curves local relations between elastic recoil pressure of the lung (Pel) and maximal expiratory flow (V̇max) were calculated and compared with the experimentally determined Pel–V̇max curve for the entire airway [illegible] all parts in series. Theory and experiments showed that the latter was the lower borderline of all the local Pel–V̇max curves. This means that the maximal flow through the entire airway at [illegible] given Pel is determined by the segment of the airway having the smallest V̇max, just as the maximal strength of [illegible] chain is determined by its weakest link. The relation between the critical transmural pressure (Ptm′) and the corresponding cross-sectional area (A′) was derived from the experimental Pel–V̇max curve. This Ptm′–A′ curve had [illegible] composed appearance, which was found to reflect parts of the different local Ptm–A curves and transitions between them because of movement of the flow limiting site within the airway. The Ptm′–A′ curve depends on the elastic properties of the flow limiting segment, and the slope of this curve (dA′/dPtm′) is the compliance of the flow limiting segment. Significant frictional pressure losses upstream from the site of flow limitation caused underestimation of both A′ and dA′/dPtm′ but downstream pressure losses had no influence on the Ptm′–A′ curve.

Key words: Mechanics of the expiration, airway compliance, maximum flow—static recoil curves, model experiments

A functional relation has been shown t[illegible] exist between transpulmonary pressure, lung inflation, and respiratory gas flow (Fry and Hyatt 1960). Graphically this relation can be described by a three-dimensional surface, on which the expiratory isovolume pressure-flow curves at least at the lower lung volumes present maxima. This means that increasing the transpulmonary pressure at a given lung volume, the flow will increase t[illegible] maximal value and then remain constant, or decrease slightly for further increase in the transpulmonary pressure.

During a forced vital capacity manoeuvre the transpulmonary pressure usually exceeds that is necessary for maximal expiratory flow during the lower 60–80 % of the vital capacity

EKLÖF, B., N. A. LASSEN, L. NILSSON, K. NORBERG, B. K. SIESJÖ and P. TORLÖF: Regional cerebral blood flow in the rat measured by the tissue sampling technique: a critical evaluation using four indicators: C^{14}-antipyrine, C^{14}-ethanol, H^3 water and $Xenon^{133}$. *Acta physiol. scand.* 1974. *91*. 1–10.

FUXE, K., Evidence for the existence of monoamine neurons in the central nervous system. IV. The distribution of monoamine terminals in the central nervous system. *Acta physiol. scand.* 1965. *64*. Suppl. 247. 37–85.

HARDEBO, J. E., L. EDVINSSON, B. FALCK, M. LINDVALL, CH. OWMAN, E. ROSENGREN and N.-AA. SVENGAARD: Experimental models for histochemical and chemical studies of the enzymatic blood–brain barrier for amino precursors. In: J. Cervós-Navarro and F. Matakas (eds.) *The Cerebral Vessel Wall*. Raven Press, New York. 1976. p. 233–244.

HORST, W. D., G. BAUTZ, E. RENYI and N. SPIRT: Inhibition of dopa accumulation by rat brain at critical L-dopa circulating concentrations. *Adv. Neurol.* 1974. *5*. 369–373.

JONSSON, G.: Quantitation of fluorescence of biogenic amines demonstrated with the formaldehyde fluorescence method. *Prog. Histochem. Cytochem.* 1971. *2*. 299–334.

LINDVALL, O. and A. BJÖRKLUND: The organization of the ascending catecholamine neuron systems in the rat brain as revealed by the glyoxylic acid fluorescence method. *Acta physiol. scand.* 1974. Suppl. 412. 1–48.

MARS, H.: Modification of levodopa effect by systemic decarboxylase inhibition. *Arch. Neurol.* 1973. *28*. 91–95.

MILLER, E. M. and L. WIENER, Ro 4-4602 and levodopa in the treatment of parkinsonism. *Neurology* 1974. *24*. 482–486.

OWMAN, CH., L. EDVINSSON, B. FALCK and K. C. NIELSEN, Amine mechanisms in brain vessels, with particular regard to autonomic innervation and blood-brain barrier. In: J. Cervós-Navarro (ed.) *Pathology of Cerebral Microcirculation*. Walter de Gruyter. Berlin, New York. 1974. p. 184–200.

OWMAN, CH. and E. ROSENGREN, Dopamine formation in brain capillaries—an enzymic blood-brain barrier mechanism. *J. Neurochem.* 1967. *14*. 547–550.

RAPOPORT, S. I.: *Blood-Brain Barrier in Physiology and Medicine*. Raven Press, New York. 1976.

TORRE, J. C. DE LA, and S. MULLAN: Experimental evaluation of L-dopa penetration in brain. *Trans. Amer. neurol. Ass.* 1971. *96*. 227–229.

WADE, L. A. and R. KATZMAN, Rat brain regional uptake and decarboxylation of L-dopa following carotid injection. *Amer. J. Physiol.* 1975. *228*. 352–359.

ed

$$\frac{dA}{dPtm} = \frac{A}{2cVmax^{3/2}} \quad (3)$$

In these equations Pel is the elastic recoil pressure of the lungs. In case of significant frictional pressure [illegible] from the alveoli to the cross-sectional area A, where the transmural pressure is Ptm, the equations still valid if we substitute Pel–Pfr for Pel, Pfr being these pressure losses. V is the flow. Vmax is the maximal flow, c constant depending on the density of the gas and the contraction coefficient (Cc) defined by the equation $A_j = Cc \cdot A$, where A_j is the area of the jet passing through A (Streeter 1966). In case of turbulent air at 25 C we get c = 0.603 (Cc)² g cm⁻⁴ s², where V is measured in l s⁻¹, A in cm² and the pressures in cm H_2O (Pedersen and Nielsen 1976). dVmax/dPel' is the slope of the curve describing Vmax as a function of Pel. dA/dPtm' is the slope of the airway compliance curve describing A as a function of Ptm. The notation (') indicates that the parameter is valid where and when the flow is maximal in the airway. At V = Vmax we have that Pel = Pel'. The notation (') is therefore not necessary in this case. If V = Vmax the measured Pel = Pfr' only if the tip of the pitot-static tube is at the flow limiting site. In the following, Pfr indicates the closest measured approximation to Pfr'.

Equation 2 states that it is possible to calculate A at any value of Vmax. If the flow is turbulent and the curve describing Vmax as a function of Pel is known, because A is determined by Vmax and the slope of this curve, dVmax/dPel at the same Vmax. The corresponding Ptm' can next be calculated from equation 1 solved for Ptm, and with Vmax, and A substituted for V and A. Accordingly we can transform the Pel–Vmax curve into a Ptm'–A curve. The relationship between Ptm' and A is determined by the elastic properties of the flow limiting segments of the airway not necessarily located at a fixed site.

Equation 3 states that we can calculate Vmax at any value of A if the Ptm'–A curve is known, because Vmax is determined by A and the slope of the curve, dA/dPtm' in the same point. The corresponding value of Pel can next be calculated from equation 1 by substituting A, Vmax and Ptm' for A, V and Ptm. We can therefore also transform the Ptm'–A curve into a Pel–Vmax curve.

In the previous paper it has been shown that it was possible to locate a point in the collapsible airway of a mechanical model of the lungs where measured relationship between Ptm and A, substituted into equation 1, gave values of Vmax equal to measured values, at least during part of the hypothetical expiration where V = Vmax throughout.

In this study we intend to show that if frictional pressure losses can be neglected (i.e. Pfr = 0) then flow limitation takes place at the particular point in the airway where the measured local Ptm–A curve, transformed into a Pel–Vmax curve by means of equations 3 and 1 for a given Pel gives the smallest Vmax of all such transformations along the collapsible airway.

We furthermore intend to show that the Ptm'–A curve calculated from the relationship between Pel and Vmax, which is the same at all points in the airway is composed of fractions of different local Ptm–A curves, each contributing to flow limitation during part of the expiration, and transitions between those.

Finally, we intend to examine how this curve is affected by changes in the resistance upstream and downstream from the flow limiting site in the airway.

As in the previous study we only examine the conditions in a collapsible airway during forced expirations before and after flow is just maximal, whereas the conditions after the maximal flow has been reached are not the object of the study. In the present paper Vmax always means just maximal flow.

Methods and Material

The experimental set-up has been described in detail in the previous paper (Pedersen and Nielsen 1976). Briefly, the lung model consisted of an air-tight transparent box containing one pair of spring-loaded bellows connected to the exterior of the box through an airway. This airway had a rigid upstream part, an elastic middle part, and a rigid downstream part. The elastic part was made of a latex finger cuff with the tip cut off, and formed into a 5 cm long converging–diverging segment with an internal diameter of 12 mm at the ends and 7 mm at the middle. At either end of the collapsible airway were slides with holes of various sizes to insert into the airway in order to vary the upstream and downstream resistances. A third slide with holes could be inserted for addition of external resistances downstream from the site, where lateral mouth pressures were measured. Lateral pressures could furthermore be measured in the bellows (i.e. "alveoli"), and in the box (i.e. the "pleural" space).

(Hyatt 1961 Mead *et al* 1967 van de Woestijne and Zapletal 1970) Expiratory maximum effort flow volume (MEFV) curves will therefore in this interval present maximal, or near maximal flows. Analysis by Fry (1958) concluded that the maximal flows were determined by the characteristics of the flow and the physical properties of the intrathoracic pulmonary system. Therefore the MEFV curve was important for diagnostic purposes, as an abnormal curve reflected intrathoracic pulmonary abnormalities (Hyatt 1965).

The physical characteristics of the intrapulmonary system determining the maximal flow have therefore been a matter of interest (Mead *et al* 1967 Pride *et al.* 1967 Fry 1968, Pardaens *et al* 1972, Clément *et al* 1973 a Lambert and Wilson 1973). From these studies it can be concluded that the elastic properties of the airway the airflow resistance in the peripheral part of the airway and the elastic recoil pressure of the lungs, closely related to the lung volume (Stead, Fry and Ebert 1952) are all important determinants of the maximal flow

In humans the relations between maximal flow and lung volume on the one hand and static elastic recoil pressure and lung volume on the other can be obtained, and construction of maximum flow-static recoil (MFSR) curves is therefore possible (Mead *et al.* 1967) Assuming that static and dynamic recoil pressures are equal, the resistance in the part of the airway upstream from the equal pressure point (EPP) can be determined from these curves (Mead *et al* 1967) or the resistance upstream from the flow limiting segment of the airway and the transmural pressure of the airway at the site of flow limitation can be determined if the latter can be regarded as a constant (Pride *et al* 1967). This is probably not the case (Pedersen and Nielsen 1976)

The purpose of the present investigation is to evaluate the MFSR curve, or rather the relationship between the dynamic elastic recoil pressure of the lungs and the maximal expiratory flow in terms of the relation between the transmural pressure of the airway where and when the flow is maximal and the corresponding cross-sectional area

It is an extension of a previous work dealing with the critical transmural pressure of the airway (Pedersen and Nielsen 1976)

The theory will be briefly reviewed and evaluated in expts. with a mechanical model of the lungs.

Theory

It is assumed that the elastic properties of the flow limiting segments of the a rway can be described by the elastic properties of a single airway behavi g exactly the same way

It is assumed that the flow is turbulent (*f* below), and the lateral pressure drop from the alveoli to location in the airway with the cross-sectional area A is solely caused by convective acceleration of the molecules, ha i g a linear velocity of zero cm the lveol nd large velocity when passing through

It is furthermore assumed that a maximal exp ratory flow occurs at y value of the elastic recoil pressure of the lungs when the transmural pressure t some point in the rw y reaches critical alue, hich less tha the elastic recoil pressure.

In that case the following set of equations ca be derived (Pedersen nd Nielsen 1976)

$$P_{el} \quad \frac{\;}{A} \dot{V}^{2} \quad P_{tm}$$

$$\frac{d\dot{V}_{max}}{dP_{el}} = \frac{A^{3}}{2c\dot{V}_{max}}$$

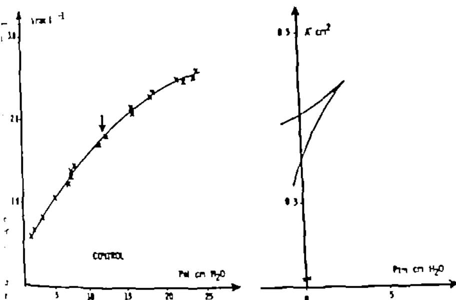


Fig. 1 *Left* The relation between elastic recoil pressure (Pel) and maximal expiratory flow (Vmax) in experiments with different initial tank pressures and/or different sizes of the external stenoses in the control situation. Crosses are experimental points, and the curve through them is fitted by eye. *Right* The derived relation between the critical transmural pressure (Ptm') and the corresponding cross-sectional area (A') (cf. text). During the expiration ... ascend along the upper branch of the angle towards the top point corresponding to the arrow at the Pel–Vmax curve and then ... descend along the lower branch.

collapsible airway. Both in the downstream part of the airway (at position A) and in the upstream part (at position C) the cross-sectional areas at given Ptm values are greater than those measured closer to the mid point of the segment (at positions B_I and B_{II}). This is because the segment is converging–diverging.

In the previous paper it was shown that the part of the Ptm–A curve visualized in expirations with just maximal flow depended on Pel at full inflation of the bellows of the mechanical model. The same curve, however, could be obtained from the time course of Pca, Ptm, and V̇ before flow during expiration reached maximal values, and in that case it could be extended towards larger Ptm values. The Ptm–A curves for positions B and B_{II} were in this way extended slightly by including points where $\dot{V} < \dot{V}max$.

It appears from Fig. 2 that these two curves are slightly different. At large Ptm values the cross-sectional area is smallest at B_I, but at low Ptm it is smallest at B_{II}. The reason for this could be an inhomogeneity of the elastic airway which was not anticipated during construction.

The curves in Fig. 2 were transformed into the corresponding Pel–V̇max curves (or (Pel–Pst(r))–V̇max curves if Pst(r) ≠ 0, *cf.* above) by use of equations 3 and 1. These curves are shown in Fig. 3. They are the Pel–V̇max curves we would get if the airway at the respective points in the elastic airway were indeed flow limiting and Pst(r) = 0. It is seen that at high Pel the airway at position B_I has the smallest V̇max, whereas at low Pel it is the airway at

In order to determine the elastic properties of the collapsible segment *in situ*, i.e. the relationship between Ptm and A, a pitot-static tube was inserted into the airway. Simultaneous measurements of the pressure difference between the end hole and the side hole of this tube (Pca − Ptot − Plat), the transmural pressure (Ptm = Plat minus pleural pressure, Ppl) and the flow through the airway (V), made it possible to determine the cross-sectional area corresponding to the tip of the pitot-static tube (*cf.* Pedersen and Nielsen 1976). Then A could be plotted as a function of Ptm. By moving the tip of the pitot-static tube, such plots could be done for different points in the collapsible segment, representing the local Ptm–A relationships.

Forced expirations were produced after maximal inspiration by rendering the box pressure positive to a degree depending on the initial pressure in a pressure tank suddenly connected to the box. The initial tank pressure and the aperture of the externally applied stenosis would then determine the effort, i.e. the transpulmonary pressure with which the expiration would be performed.

During forced expirations with different initial tank pressures (10, 20, and 40 mmHg) without and with externally applied stenosis (8 mm, 6 mm, 5 mm, and 4 mm in diameter), the time course of V, Ptm, and Pca, was recorded in one series of experiments and the time course of V and Pel in another otherwise identical series. On these curves just maximal flows were detected as described previously (Pedersen and Nielsen 1976) and corresponding values of Vmax, Ptm, Pca, and Pel were read on the curves. For a given position of the pitot-static tube a Ptm–A curve valid for V = Vmax and a Pel–Vmax curve were constructed. Four positions were chosen for the tip of the pitot-static tube: position A was in the downstream part of the collapsible segment, positions B_I in the middle, position B_{II} a little above the middle, and position C in the upstream part of the collapsible segment.

By use of the 4 different Ptm–A curves, equations 3 and 1 (Cc = 1.03 for the converging part of the airway and Cc = 0.77 for the diverging part of the airway (Pedersen and Nielsen 1976), the corresponding Pel–Vmax curves (or rather (Pel–Psfr)–Vmax curves, *cf.* above) were calculated. These curves express according to our theories the Pel–Vmax relations we would obtain, if the segment with the given Ptm–A relation were indeed flow limiting and Psfr = 0. These calculated local curves were then compared with the experimental Pel–Vmax curve for the entire airway to see if there were any concordance.

Some of the experiments were repeated after inserting a stenosis upstream or downstream into the rigid part of the airway so that the diameter of the airway was reduced by approximately 50 %. The purpose was to see whether a change in the upstream or downstream resistance would affect the relationship between Pel and Vmax and the derived Ptm–A relationship.

Results

If the mechanical model should be in accordance with the theoretical model on which the derived equations were based, significant frictional pressure losses (Psfr) between the alveoli and the cross-section A should be included in the equations by substituting Pel–Psfr for Pel. As shown in the appendix, section I, Pel–Psfr is equal to Pca + Ptm and could therefore be calculated from the tracings, and so could Psfr for known values of Pel. With no stenosis inserted into this part of the airway, Psfr was usually small and could be neglected.

Fig. 1 (left part) shows the relation between Pel and Vmax without insertion of any upstream or downstream stenosis into the airway. The crosses are experimental points, and the curve through them is drawn by eye. The curve is transformed into the corresponding Ptm–A curve by use of the equations 2 and 1. The transformed curve is shown in the right part of the figure. At the highest value of Vmax (and Pel) the relation between Ptm and A gives a point to the far left on the upper branch of the transformed curve. As the expiration proceeds, Ptm and A increase and we ascend along the upper branch towards the top point of the angle (corresponding to the arrow at the Pel–Vmax curve). As the flow decreases further we move back again along the lower branch of the angle, with decreasing values of Ptm and A.

Fig. 2 shows the measured relationships between Ptm and A at the four points in the

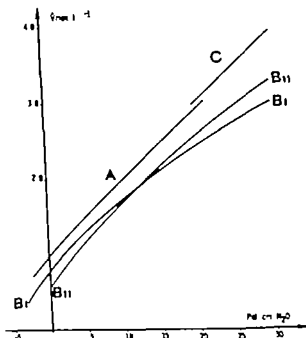


Fig. 3 Relations between elastic recoil pressure (Pel) and maximal expiratory flow (Vmax) calculated from the corresponding curves in Fig. 2 by use of equations 3 and 1.

To see if this were the case in the present experiments, the experimental points of Fig. 1 upper part, were plotted in a diagram showing the lower borderline of the curves in Fig. 3. The result is shown in Fig. 4 (left part). The curve to the right of the arrow is part of B_I, and the curve to the left is part of B_{II} in Fig. 3. The experimental points and the theoretical curves appear indeed to agree fairly well, although the experimental points are placed slightly to the right of these curves. This may be due to Psfr 0, as Psfr is the horizontal distance between a (Pel–Psf)–Vmax curve and a Pel–Vmax curve.

The Ptm–A curve has a course that cannot be immediately explained by the different local Ptm–A curves (compare Fig. 1 right part, with Fig. 2). The descending portion is in harmony with part of B_{II}, but the ascending portion has apparently no correlate. If we assume that the composed theoretical curve in Fig. 4 left part, is indeed the actual Pel–Vmax curve, then the corresponding Ptm–A curve must be made up by parts of the curves B_I and B_{II} in Fig. 2, because the theoretical curve was derived from these two curves. The question about which parts of the curves are involved, can be solved by use of equations 2 and 1 with substituted Vmax and dV̇max/dPel valid for the end points of the curve sections B_I and B_{II} in Fig. 4 left part. The right part of Fig. 4 shows sections of the curves B_I and B_{II} from Fig. 2, but only the full-line fragments of these curves are involved in flow limitation. During the hypothetical expiration where V = Vmax throughout, we proceed from right to left on curve B_I. Reaching the arrow at B_I, the site of flow limitation suddenly shifts a little further upstream, where the curve B_{II} is valid. There is a discontinuous jump between the two arrows both corresponding to the arrow at the Pel–Vmax curve. After this jump we move down curve B_{II} as the expiration proceeds. The discontinuous jump is due

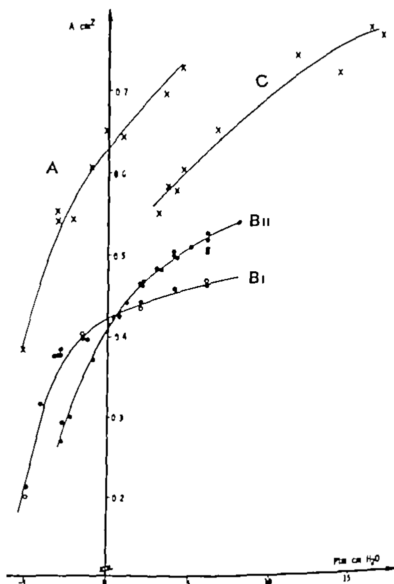


Fig. 2. Measured relations between transmural pressure (Ptm) and cross-sectional area (A) at 4 positions of the pitot-static t be in the collapsible segment. Position A is in the downstream part of the segment. Position B_I is the middle, position B_{II} is little bove the middle, a d position C is in the upstream part of the segment. The crosses, circles, and f lled-out circles are experimental points. The curves through them are f tted by eye

position B_{II} The change of Pel from positive to negative values at low Vmax means that the elastic forces tend to expand, rather than constrict the "lung" This is only possible, if the airway can resist a negative transmural pressure without collapsing.

As the flow is identical in all parts of the airway (flow caused by airway collapse is assumed to be negligible) the maximal flow must be determined by the part of the airway with the smallest Vmax, just as the strength of a chain is determined by its weakest link. The Pel-Vmax curve for all parts in series must therefore be the lower borderline of all curves each describing local flow maxima at different points along the elastic airway

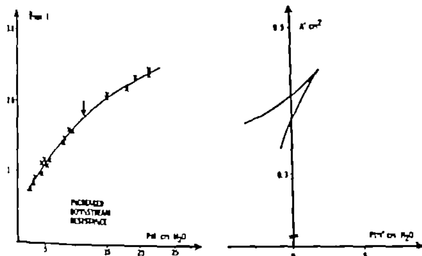


Fig 5 *Left* The relation between elastic recoil pressure (Pel) and maximal expiratory flow rate (Vmax) in experiments with increased downstream resistance. *Right*. The derived relation between the critical transmural pressure (Ptm') and the corresponding cross-sectional area (A') Compare with the control situation Fig. 1.

curves in Fig. 1 and 5. The position of the latter is not changed, but it is shorter corresponding only to the lower half of these curves. By means of equations 2 and 1 the curves were transformed into the corresponding Ptm–A curves. In the latter case Pel–Pzf was substituted for Pel. The result is shown in the right part of the figure. If Pel–Pzf is substituted for Pel, the Ptm–A curve has a course which is similar to the descending branch of the Ptm–A curves in Fig. 1 and 5 If Pzfr is neglected and Pel is substituted into the equations 2 and 1 A is underestimated at all Ptm' as expected (*cf* above). It is furthermore seen that the slope of the curve, *i.e.* the airway compliance, is underestimated. Therefore, the airway appears to be more stiff than it actually is.

Besides, the curve is shifted slightly to the left, which means that Ptm is underestimated by neglecting Pzfr Pzf was measured as the horizontal distance between the curves in the left part of the figure, and log Pzfr plotted as function of log Vmax in Fig. 7 The best fitting straight line has the slope 2.58, which agrees with the finding that Ptm' is underestimated (*cf* appendix, section II)

Discussion

In these experiments we measured the relations between transmural pressure and cross-sectional area at 4 positions in a converging–diverging elastic airway of a mechanical model of the lung. Four local Ptm–A curves were drawn and transformed into corresponding Pel–Vmax curves, which were compared with the measured, actual Pel–Vmax curve, obtained with the tip of the pitot-static tube placed little above the middle in the airway (at position B_{II}). This comparison is justified only if frictional pressure losses can be neglected as in most of these experiments and if the local Ptm A curves are not influenced by the

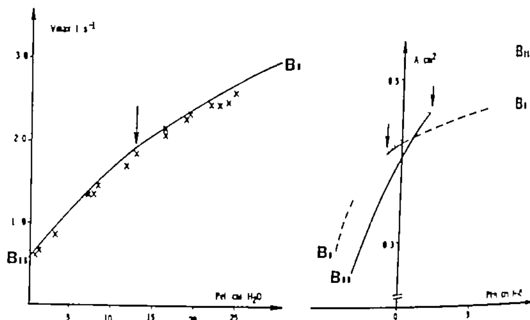


Fig. 4. *Left* The experimental points f Fig. 1 left part, plotted i diagram showing the lower border line of the curves i Fig. 3 This is composed of the curve sections B_I and B_{II} t each side of the ... sect on (ma ked by the arrow). *Right* Here B_I a d B_{II} are parts of the corresponding curves in Fig 2 but only the f ll-line fragments are derived from the curve sections above During the expiration pro ceed down the full-line fragment of B_I Reachi g the rrow we jump t the arrow t B_{II} and continue do this curve. The jump between the two arrows both corresponding t the one arrow at the Pel–Vmax curv bove is i dicated by the dotted curv

to the fact that the curve sections B_I and B_{II} in Fig 4 left part, form an obtuse angle a the arrow The best fitting curve (Fig. 1 left part) has a smooth course, and therefore the Ptm –A curve (Fig. 1 right part) shows no discontinuity Its ascending portion may therefore reflect the upstream motion of the flow limiting site (*i.e* motion from B_I to B_{II}) as indicated by the dotted curve between the two arrows in the right part of Fig. 4.

As downstream pressure drops do not appear in equations 1 2 and 3 no change in these relations were expected after insertion of a downstream stenosis. This is confirmed by Fig 5 which shows the effect of an increased downstream resistance on the Pel–Vmax curve (left part of the figure) and on the derived Ptm –A curve (right part of the figure) Compared with Fig. 1 there are no marked differences. But no differences were expected either

Insertion of an upstream stenosis increased Psfr which could not be considered negligible any more. Therefore Pel–Psfr should be substituted for Pel in equations 1 and 2, if they should still be valid If we neglect the influence of Psfr which presumably increases with Vmax, then A and dA /dPtm will both be underestimated as shown in the appendix, section II It is here furthermore shown that Ptm may be overestimated or underestimated depending on the relation between Vmax and Psfr

To check this hypothesis, Ptm –A curves were constructed by substitution of both Pel and Pel–Psfr into equations 1 and 2 in the experiments with increased upstream resistance.

Fig. 6 shows the effects of an increased upstream resistance. In the left part of the figure are shown two curves. One is the Pel–Vmax curve, and the other gives the relation between Pel–Psfr and Vmax. The former is shifted to the right compared with the corresponding

lower borderline of the four curves. Stepwise withdrawal of the tube from B_{II} to B_I and further to A will affect the Ptm–A relations at these positions in a similar way but it is difficult to tell how this will influence the flow limiting properties of the airway.

The effect of complete removal of the pitot-static tube from the collapsible part of the airway was a slight increase of Vmax at all Pel values, and a corresponding increase of A at all Ptm values, with actually no change of Ptm. The increase of A was about 0.07 cm and this equalled almost exactly the cross-sectional area of the pitot-static tube, which determined the part of the cross-sectional area available for airflow *i.e.* around the tube. The fact that this increase of A was the only effect of removal of the pitot-static tube indicates that the behaviour of the collapsible segment was not influenced by the presence of the tube.

The concordance between the measured points and the calculated Pel–Vmax curve fraction giving the smallest Vmax at a given Pel (*cf* Fig. 4 left part) indicates that the segment of the airway with the smallest Vmax at a given Pel, is the flow limiting segment, and we find no evidence that the presence of the pitot-static tube interferes with this conclusion.

In the present experiments it was not the same segment that was flow limiting at all Vmax values. Therefore, the Ptm–A curve derived from the measured Pel–Vmax curve was not identical with any particular local Ptm–A curve, but had a composed appearance with an ascending and a descending branch (*cf* Fig. 1 right part). The further analysis indicated that the ascending branch coincided with motion of the flow limiting site from one part of the segment to another with a different Ptm–A relation. The descending part, however corresponded closely to the Ptm–A relation at this other part of the segment.

The factors determining which airway segment is flow limiting may be evaluated. Both the cross-sectional area A and the airway compliance dA/dPtm at the same A are determinants (*cf* equation 3). Fig. 4 right part, shows that the cross-sectional area at the left arrow is smaller than that at the right arrow. The motion of the flow limiting site from B_I to B_{II} along the dotted curve involves that flow limitation does not take place at the smallest cross section any more.

If the site of flow limitation moves to a segment with a larger cross-sectional area, the same Vmax can only be achieved if also the compliance of the airway dA/dPtm is larger at the new site. This is stated in equation 3, and is illustrated in Fig. 4 right part, where the points corresponding to the two arrows both satisfy equation 3 for the same Vmax (1.9 l s⁻¹)

The present experiments were intended to elucidate the conditions at just maximal flows. We therefore only described conditions which may be assumed to correspond with the curve through the points of maximal flow on the three-dimensional surface of Fry and Hyatt (1960) described by way of introduction. Flows at larger transpulmonary pressures than necessary for just maximal flow may still be maximal in the sense that they are determined by equation 3, solved for Vmax, but the local Ptm–A curves may then be changed, due to downstream compression of the collapsible airway (Pedersen and Nielsen 1976). An elucidation of these conditions is beyond the scope of the present study.

As the ultimate purpose of the present investigation is to explain the MFSR curves obtained in experiments with humans, we will now discuss the results from this point of view. In the human peripheral airway frictional pressure losses may possibly not be neglected.

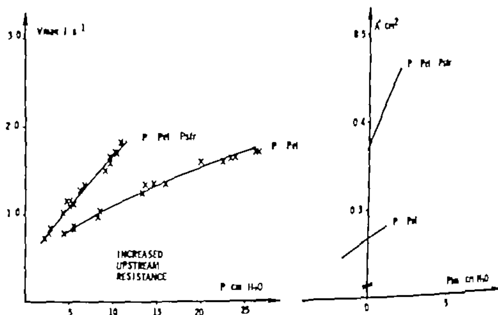


Fig. 6. *Left* The relation between elastic recoil pressure (Pel) and maximal expiratory flow rate (Vmax) ... expts. with increased upstream resistance. Vmax is also shown as a function of the elastic recoil pressure ... plus the pressure loss between the bellows and the tip of the pitot-static tube at position B_{II} (Pel-Psfr). The crosses are experimental points and the curves through them are fitted by eye. *Right* The relation between the critical transmural pressure (Ptm') and the corresponding cross-sectional area (A') derived from the two curves above (*cf* text). Compare with Fig. 1 and 5

presence of the pitot-static tube by means of which they were measured and which had to be moved from one position to another between the experiments.

Curve C in Fig. 2 was measured with the tip of the pitot-static tube at position C. We cannot tell exactly what the Ptm –A relation at C will be, when the tube is withdrawn to position B_{II} which is our point of reference, but the cross-sectional area available for airflow at position C will increase as the area occupied by the tube is added, whereas the cross-sectional areas at the further downstream positions A, B_I and B_{II} are expected to remain unchanged because the tip of the tube has approximately the same diameter as the tube itself. Curve C in Fig. 2 would therefore be displaced upwards, and so would the Pel Vmax curve calculated from it. Such a displacement of curve C in Fig. 3 would not change the

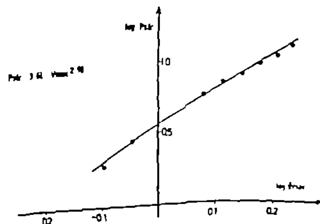


Fig. 7. The logarithm to Psfr measured at regular flow rates is as the horizontal distance between the two curves in the upper part of Fig. 6, plotted as a function of log Vmax. The best fitting line through the points is given by the equation on the figure

ner borderline of the four curves. Stepwise withdrawal of the tube from B_{II} to B_I and rder to A ill affect the Ptm–A relations at these positions in a similar way but it is ifficult to tell how this will influence the flow limiting properties of the airway

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As the ultimate purpose of the present investigation is to explain the MFSR curves ob tained in experiments with humans, we will now discuss the results from this point of view In the human peripheral airway frictional pressure losses may possibly not be neglected.

Therefore we examined what the curves would look like if upstream frictional pressure losses (Psfr) were neglected after insertion of an upstream stenosis. We found that the cross sectional area of the flow limiting segment was underestimated, and that this was also the case with the compliance of the flow limiting segment, *i.e.* the slope of the Ptm–A curve. The critical transmural pressure, however, was not much changed because Psfr varied almost proportional to Vmax squared.

In the model, the upstream stenosis was independent of the lung volume, and therefore had a constant cross-sectional area. In that case Psfr should vary with $\dot{V}max^2$. This was not far from what we found. In humans, the linear dimensions of the peripheral airways vary with approximately the cube root of the thoracic gas volume (Hughes *et al.* 1972). During the expiration the dimensions of these airways therefore decrease, and the exponent n in the equation $Psfr = k_{sfr} \dot{V}max^n$ will even in case of complete turbulent flow probably be less than 2 (Pedersen 1973, p. 102 ff.). Neglection of Psfr, therefore, will probably cause Ptm to be overestimated (*cf.* appendix, section II).

The unique relation between maximal expiratory flow and elastic recoil pressure or lung volume has been challenged by several authors. As pointed out by Mead *et al.* (1967) the maximal flow will depend also on time in case of unequal ventilation of different lung units. In a monalveolar lung model the maximal flow measured at the mouth will be time dependent too, due to flow from the collapsing airway (Clément *et al.* 1973 b) and airway wall resistance or inertia (Clément *et al.* 1974).

In the mechanical model we assumed that time factors could be neglected. This was probably true for several reasons. Firstly, it was a monalveolar model, and no unequality of ventilation could exist. Secondly, the volume of the collapsible airway was very small, and airway flow therefore minimal. Airway flow is expected to be largest when the rate of change of transpulmonary pressure is large (Clément *et al.* 1973 b). For these reasons airway flow could only be expected to contribute at high Vmax in the present experiments due to this gradient otherwise being small. Finally, inertia and resistance of the wall of the collapsible segment could probably be neglected, as this was very thin.

By application to the lungs of the theories evaluated here, these factors may not be negligible and must therefore be included in the considerations.

Appendix

Section I

As defined in the text,

$$Pes \quad Ptot - Plat \qquad (I\text{-}1)$$

$$Ptm \quad Plat - Ppl \qquad (I\text{-}2)$$

By addition,

$$Pes \quad Ptm \quad Ptot - Ppl \qquad (I\text{-}3)$$

As Psfr is the difference between the pressure inside the bellows (Palv) and the end-hole pressure of the pitot-static tube,

$$Psf = Palv \quad Ptot \qquad (I\text{-}4)$$

Substituting Palv = Pel + Ppl, and rearranging,

$$Ptot \quad Ppl \quad Pel \quad Psf \qquad (I\text{-}5)$$

[illegible] (I-3) and (I-5),

$$\mathrm{Pcn} + \mathrm{Ptm} = \mathrm{Pel} \quad \mathrm{Psf} \tag{I-6}$$

[illegible] II

If there [illegible] significant upstream frictional pressure loss, Psf then Pel–Psf should be substituted for Pel [illegible] equation 1 [illegible] the text, and [illegible] get:

$$\mathrm{Pel} - \mathrm{Psfr} = \frac{}{A} V^{2} + \mathrm{Ptm} \tag{II-1}$$

If we neglect Psfr, equation II-1 may be [illegible] written

$$\mathrm{Pel} = \frac{}{A^{\prime}} V^{2} + \mathrm{Ptm}_{n} \tag{II-2}$$

[illegible] where A_n and Ptm_n are not necessarily equal to A and Ptm.

For V [illegible] Vmax we get from equation 2 in the text

$$\frac{d\mathrm{Pel}}{d\mathrm{Vmax}} - \frac{d\mathrm{Psf}}{d\mathrm{Vmax}} = \frac{2c\mathrm{Vmax}}{A^{*}} \tag{II-3}$$

in the first case, and

$$\frac{d\mathrm{Pel}}{d\mathrm{Vmax}} = \frac{2c\mathrm{Vmax}}{A} \tag{II-4}$$

[illegible] the second case.

From equations II-3 and II-4 [illegible] see that if dPsfr/dVmax [illegible] 0, then A [illegible] A [illegible] and if dPsfr/dVmax [illegible] 0, then A_n [illegible] A. *This means that neglection of Psfr may cause A to be underestimated.*

F[illegible] V [illegible] Vmax we get from equation 3 in the text

$$\frac{dA}{d\mathrm{Ptm}} = \frac{A}{2c\mathrm{Vmax}^{2}} \tag{II-5}$$

[illegible] if Psfr [illegible] neglected

$$\frac{dA}{d\mathrm{Ptm}_n} = \frac{A_n}{2c\mathrm{Vmax}} \tag{II-6}$$

As [illegible] above, neglection of Psfr [illegible] cause $A_n < A$ if dPsfr/dVmax [illegible] 0. Because both A [illegible] and A [illegible] 0, [illegible]

$$\frac{dA_n}{d\mathrm{Ptm}_n} < \frac{dA}{d\mathrm{Ptm}} \tag{II-7}$$

which means that the compliance of the airway may be underestimated at [illegible] given Vmax, *i.e. the airway may appear more stiff than [illegible] actually is if Psfr is neglected.*

Solving II-1 for Ptm and substituting $V^{2}/A = c\mathrm{Vmax}/A^{*} = \mathrm{Vmax}(d\mathrm{Pel}/d\mathrm{Vmax} \quad d\mathrm{Psfr}/d\mathrm{Vmax})/2$ from II-3, we get for V [illegible] Vmax

$$\mathrm{Ptm} \quad \mathrm{Pel} - \mathrm{Psfr} - \frac{\mathrm{Vmax}}{2}\left(\frac{d\mathrm{Pel}}{d\mathrm{Vmax}} - \frac{d\mathrm{Psfr}}{d\mathrm{Vmax}}\right)$$

by rearranging,

$$\mathrm{Ptm} + \mathrm{Psfr} \quad \frac{\mathrm{Vmax}}{2}\left(\frac{d\mathrm{Psfr}}{d\mathrm{Vmax}}\right) = \mathrm{Pel} - \frac{\mathrm{Vmax}}{2}\left(\frac{d\mathrm{Pel}}{d\mathrm{Vmax}}\right) \tag{II-8}$$

Solving II-2 for Ptm_n and substituting cV/A [illegible] Vmax /A [illegible] Vmax(dPel/dVmax)/2 from II-4 [illegible] similarly

$$\mathrm{Ptm} = \mathrm{Pel} \quad \frac{\mathrm{Vmax}}{2}\left(\frac{d\mathrm{Pel}}{d\mathrm{Vmax}}\right) \tag{II-9}$$

From equations II-8 and II-9 we see that

$$Ptm = Ptm + Psfr - \frac{Vmax}{2}\left(\frac{dPsfr}{dVmax}\right) \quad (I$$

If $Psfr = k_{sfr} Vmax$ where k_{sfr} and n are arbitrary constants, then

$$Ptmu_n = Ptm + \left(1 - \frac{n}{2}\right) k_{sfr} Vmax \quad (I$$

For $n < 2$ nd $k_{sfr} > 0$
Ptm > Ptm *I* neglection of Psfr causes Ptm to be overestimated.

For $= 2$ and $k_{sfr} > 0$
Ptm_n = Ptm *I* neglection of Psfr does not invalidate the determination of Ptm'

For $n > 2$ nd $k_{sfr} > 0$
Ptm < Ptm *i.e* neglection of Psfr causes Ptm to be underestimated

References

CLÉMENT J J PARDAENS and K. P VAN DE WOESTIJNE, Expiratory flow rates, driving pressures and dependent factors. Simulati n by means f a model. *Respir Physiol.* 1974 *0* 353–369

CLÉMENT J K P VAN DE WOESTIJNE, and J PARDAENS, A general theory of respiratory mechanics a to f rced expiration. *Respir Physiol* 1973 b *19* 60–79

CLÉMENT J M AFSCHRIFT J P DAENS and K P VAN DE WOESTIJNE Peak expiratory flow rate as of change of pleural pressure. *Respir Physiol.* 1973 a. *18* 22. 237

FRY D L., Theoretical considerations of the bronchial pressure-flow- olume relationship ith par reference to the maximum expiratory flow volume curve. *Phys. Med. Biol* 1958. *3* 174–194.

F D L A preliminary lung model for simulating the aerodynamics of the bronchial tree. C biomed *R s.* 1968 . 111 134

FRY D L a d R E HYATT Pulmonary mechanics. A unified analysis of the relationship between pr volume and gas flow in the lungs of normal and diseased human subjects. *Amer J Med* 19 67?–689

HUGHES, J M B., F G HOPPIN, JR. and J MEAD Effect of lung inflation on bronchial length an meter l excised lungs. *J appl Physiol.* 1972. *32* .5–35

HYATT R E The l terrelationships of pressure flow and volume during various respiratory man in normal nd emphysematous subjects. *Amer Rev resp Dis* 1961 *83* 676–683

HYATT R. E., Dynamic lu g volumes In *Handbook of Physiology* Section 3 Respiration. Vol American Physiological Society Washington D C 1965 1381 1397

LAMBERT R. K. and T A. WILSON, A model f the elastic properties of the lung and their effect on tory flow *J appl Physiol* 1973 *34* 34–48

MEAD, J J M TURNER, P T MACKLEM d J B LITTLE, Significance of the relationsh p betwee recoil and maximum expiratory flow *J appl Ph siol* 1967 *22* 95–108

PARDAENS, J K. P AN DE WOESTIJNE and J CLÉMENT A physical model of expiration. *J appl. P* 1972. *33* 479–490.

PEDERSEN, O F *The mechanics f he xpiration evaluated b model* Thesis, Marselisborg Hos Institute of Physiology Århus 1973 (Summarized i *Act ph) siol. scand.* S pplementum 386. May be obtained free of charge from the thor as long as copies are va lable)

PEDERSEN O F and T M NIELSEN, The critical transm ral pressure of the irway *Acta ph siol* 1976. *97* 426–446.

PRIDE, N B., S. PERMUTT R L RILEY and B BROMBERGER BARNEA, Determinants of maximal expi flow from the lungs. *J appl. Physiol* 1967 *23* 646–662.

STEAD W W D L FRY and R V EBE T The elastic properties of the lung in normal men and tients with chronic pulmonary emphysema *J Lab clin Med.* 1952. *40.* 674–681

STREETER, V L *Fluid Mechanics*, 4th edition McGraw Hill Book Company N Y 1966, p 420.

VAN DE WOESTIJNE, K. P and A. ZAPLETAL, The maximum expiratory flow-volume curve. Peak flo effort independent portion. In *Airway D namics—Ph siolog and Pharmacology* edited by A. Bo Thomas, Springfield, Ill. 1970. 61 72.

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From the Institute of Biological Chemistry A, Copenhagen, Denmark

Effect of the Polyene Antibiotic Filipin on the Permeability of the Inward- and the Outward-facing Membranes of the Isolated Frog Skin (*Rana temporaria*)

By

ROBERT NIELSEN

Received 16 July 1976

Abstract

NIELSEN, R. *Effect of the polyene antibiotic filipin on the permeability of the inward- and the outward-facing membranes of the isolated frog skin* (Rana temporaria). Acta physiol. scand. 1977 99 399–411

The effect of the polyene antibiotic filipin on the electrical properties and the passive permeability of the cell membranes was investigated. The addition of filipin to the outside bathing solution has the following effects: 1. it results in a drastic reduction in the transepithelial resistance and potential, 2. it causes a 10–20 fold increase in the passive transepithelial chloride, sodium and sucrose flux, 3. it results in the formation of an amiloride insensitive sodium pathway in the outward facing membrane, 4. it results in an active outward transport of potassium, 5. it results in a highly significant swelling of all the cells in the epithelium. The addition of filipin to the inside bathing solution has the following effects: 1. it results in an activation of the active sodium transport, 2. it causes a slight increase in the passive transepithelial chloride and sodium permeabilities but has no effect on the sucrose permeability, 3. it has no effect on the amiloride inhibition of the short-circuit current, 4. it has no effect on the volume of the cells in the epithelium. It is suggested that the addition of filipin to the outside bathing solution increases the direct sodium flow from cell to cell in neighbour layers. Furthermore these experiments indicate that the outward facing membrane of the isolated frog skin has a high cholesterol content as compared with the cholesterol content of the inward facing membrane.

The polyene antibiotics are characterized by a large polyhydroxy lactone ring of 23–43 atoms with 4 to 7 conjugated double bonds in the ring. The polyene antibiotics filipin and amphotericin B mediate changes in the permeability of bilayer membranes, liposomes, erythrocytes, fungi and intestinal mucosal cells from chicks (for ref. see Norman *et al.* 1972).

In sodium transporting epithelia, such as the frog skin and the toad bladder, the addition of amphotericin B to the outside (the mucosal side) bathing solution of the epithelia resulted in an increase in the passive chloride and urea permeabilities, whereas the effect on the active sodium transport was variable (Lichtenstein and Leaf 1965, Finn 1968, Nielsen 1971). The addition of amphotericin B to the inside of the frog skin and the serosal side of the toad bladder had no effect on the active sodium transport (Lichtenstein and Leaf 1965

Finn 1968 Nielsen 1971) On the basis of these and other experiments Lichtenstein and Le (1965) concluded that amphotericin B increased the permeability of a dense diffusion barr near the mucosal surface to small solutes, whereas Finn (1968) concluded that amphoteric B increased the passive movement of sodium and small solutes across the membrane fac the medium to which the drug was added.

In a previous paper I have suggested a model to explain the changes in the sodium transpo during the aldosterone induced moult (Nielsen 1972, 1973) This model was partially bas on the assumption that addition of filipin or amphotericin B to the outside bathing solutio resulted in an increase in the passive transepithelial sodium flux.

In the present studies it is shown that addition of filipin to the outside bathing solutio results in a 10–20 times increase in the passive transepithelial sodium, chloride and sucro fluxes. Furthermore the addition of filipin to the inside bathing solution results in an i crease in the active sodium transport but has nearly no effect on the passive transepithel sodium chloride and sucrose fluxes.

Methods

The experiments were performed o male and female frogs (Rana temporaria). The frogs ere kept tially immersed in tap water t about 4 C. The skins were dissected from pithed animals and divided t o laterally symmetrical pieces. The skins were mou ted n perspex chambers and bathed in stirred Ring solutio (Na − 115 0 K 2.5 Ca − 1 0, HCO₃ 2.4 Cl − 117 1 mM pH − 8.2).

The short-circuit experiments were performed according to the method of Ussing and Zerahn (19 usi g an automatic voltage clamp apparatus programmed to disconnect the short-circuit current e five min tes, thus allowing the potential t be measured for 60 s The fluxes re in some experim expressed in t rms of permeability coefficients, calculated by adding the isotope to the medium bat o side and measuring its rate of appearance on the other side (Andersen and Ussing 1957).

$$P \quad (a_2 V \quad a V)/A \quad a_4 \quad (t_4 \quad t)$$

a and a_4 designate the radioact vity originat g from side 2 in one ml of solution from side 1 between th t and t_2, respectively V a d V th correspond ng olumes of the solution on side 1 A the area of membra e a d a_4 the mean activity one ml of solution from side duri g the period from t to t_4. filipin Lot u-5956 was gift from the Upjoh C mpany K lamazoo Michigan, U.S A

Results

The effect of filipin on the short-circuit current (SCC) and transepithelial potential (PD)

Addition of filipin to the outside bathing solution Fig. 1 A shows the effect of adding 5 filipin to the medium bathing the outside of the isolated frog skin, Fig. 1 B shows the cont skin half 10–40 min after the addition of 5 μM filipin the PD and resistance started decrease Higher concentrations of filipin (50 μM) caused the decrease in PD and resista to start 2–3 min after the addition of the filipin 0 5 μM had no effect on the PD and resistance 5 μM filipin caused a 40–75 % reduction in the PD 50 μM filipin caused a 75–9 reduction in the PD The effect of filipin on the SCC was variable In about half of experiments filipin had no effect on the SCC or caused 10 50 reduction in the SCC the remaining experiments filipin caused 10–100 activation of the SCC. This activat of the SCC lasted 1–2 h.

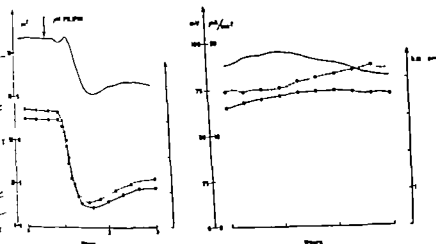


Fig. 1A and B. Addition of filipin to the outside bathing solution. Effect of filipin on the short-circuit current and potential across the frog skin. A. At the arrow filipin was added to the outside to give concentration of 5 µM. B. Control skin half. —— short-circuit current (µA/cm²); O—O potential (mV); ●—● resistance (Kohm cm²).

Kinsky (1963) has shown that 3 µM filipin caused complete hemolysis of rat erythrocytes, while 1 µM no hemolysis occurred. Van Zutphen *et al.* (1971) have shown that 0.4 µM filipin caused about 95% reduction in the resistance of lecithin-cholesterol (1/1) bilayer membranes. In the presence of 0.4 µM filipin the lecithin cholesterol bilayer membrane was stable, in the presence of 4 µM filipin the survival time of the membrane was about 6 min, and in the presence of 40 µM the survival time of the membrane was less than 1 min (Van Zutphen *et al.* 1971). Thus the concentration of filipin that induced a reduction in the PD and the resistance across the isolated frog skin, hemolysis of rat erythrocytes, and a decrease in the survival time of bilayer membranes was the same. Therefore the decrease in PD and resistance in the isolated frog skin after the addition of filipin to the outside may be due to partial disintegration of the outward-facing membrane.

Addition of filipin to the inside bathing solution. Addition of 5 µM filipin to the inside had no effect on the PD and the SCC. About 5 min after the addition of 50 µM filipin the SCC and to a lesser degree the PD increase (Fig. 2 A). The increase in SCC was 10–100% of the control level. In half the expts. the current response to filipin addition was triphasic, the first maximum in SCC occurring 20–45 min after addition of filipin; thereafter the SCC started to decline to about the control level. After this decrease the SCC increased again and reached its second maximum after about 2 h of incubation.

The effect of filipin on sodium flux

To ascertain whether the increase in the SCC after addition of filipin to the inside of the frog skin was due to an increase in the active sodium flux, a series of double labelling expts. was performed. The net sodium flux and the SCC were identical both in the control period and in the two periods after the addition of filipin (Table I). Thus, the addition of filipin to

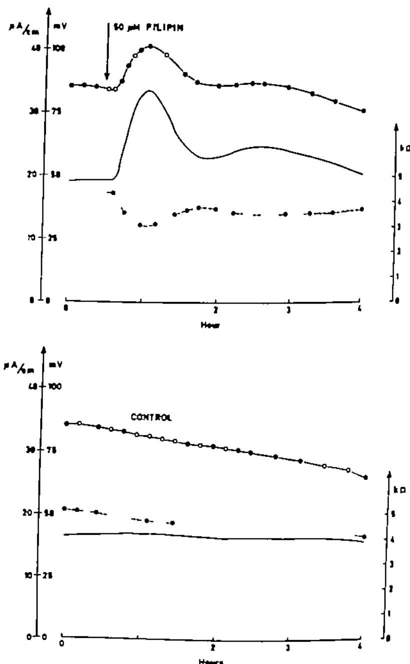


Fig. 2 A and B. Add tion of filipin t the nside bathing solution. Effect of f lipin on th current and potential across the frog skin A At the arrow filipin was added t the inside centration of 50 µM B Control sk n half —— short-circu t current (µA/cm²) O—O p O—O resistance (Kohm cm²)

the inside bathing solution of the frog skin results in an activation of the ac transport.

Effect on passive fluxes

To evaluate the effect of filipin on the permeability of the frog skin to substances, t of which can be accounted for by passive forces, transepithelial sucrose and

Table I The net sodium flux and short-circuit current across the frog skin before and after the addition of filipin (50 µM) to the inside of the frog skin.

Period	Influx	Outflux	Net flux	SCC	
			µeq/cm² h		
Control	1 16	0 05	1 11	1 12	0 60 > p 0 50
Filipin 1 h	1.33	0 06	1.27	1.30	0 60 p 0.50
Filipin 2 h	1 26	0 04	1.22	1 19	0 50 p 0 40

The influx was measured by means of Na-22 and the outflux simultaneously with Na-24. The SCC was measured and recorded automatically. The mean results from 7 expts. are presented. Each period was 1 h.

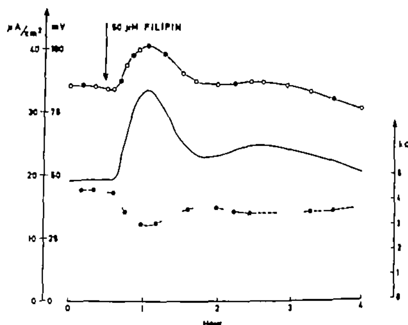
and outfluxes were determined. The sodium outflux (which is passiv) was also measured. Table II shows that the addition of filipin to the outside bathing medium, (the skins were mounted in Ringer's solution containing 1 mM sucrose) results in large and sustained increases in the influx and outflux of sucrose, whereas the addition of filipin to the inside had no effect on these fluxes. The addition of filipin to the outside bathing medium results in a large and sustained increase in the chloride in- and outflux, and in the sodium outflux (Table III). Furthermore there was no significant difference between the chloride in- and outflux. The addition of filipin to the inside bathing medium had only a small effect on the chloride in- and outflux and on the sodium outflux (Table IV). Fig. 3 shows a series of experiments in which the sodium outflux is plotted against the chloride outflux. The outfluxes of sodium and chloride were measured simultaneously on the same skin after the addition of filipin to the outside bathing medium. The slope of the line drawn in Fig. 3 is equal to the ratio of the free solution mobilities of sodium and chloride i.e. 0 66. The experimental points fit the theoretical line. This indicates that the addition of filipin to the outside bathing medium results in the formation of a nonspecific transepithelial pathway

Previous data (Nielsen 1971) have shown that the addition of 50 µM amphotericin B to the outside bathing solution results in a 5 times increase in the sodium outflux and a 5 times increase in in- and outflux of chloride and urea. The increases in the chloride and urea in- and outfluxes were essentially the same in both directions. The addition of amphotericin

Table II Effect of 50 µM filipin on the permeability of the frog skin to sucrose.

Period	Control ±S.E. A–B	Filipin ±S.E. B–C	C D	D–E
		cm sec⁻¹ 10⁻		
Filipin on outside	0 87±0 24	7 76±0.54	10 6±0 6	13.7±1 7
inside	0 61±0 07	0 57±0 11	0 59±0 10	0 70±0 09

In each expt following 20 min of equilibration for the isotope the permeability across the tissue was determined for one, 1 h control period (period A–B) Filipin was then added to the outside or the inside bathing medium to give concentration of 50 µM. After the addition of filipin solution, the permeability was determined for 3 further 1 h periods (periods B–C, C–D and D–E) Both the sucrose in- and outflux were measured in these expts. The results were essentially the same for fluxes in either directions. All values are the means ±S.E. of 6 expts.



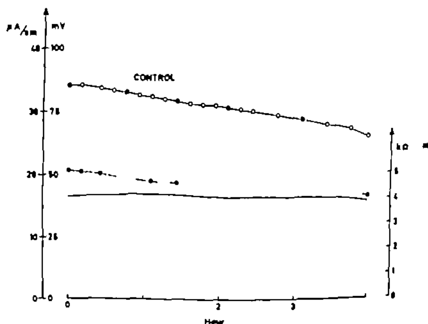


Fig. 2 A and B Add tion of f lipin to the nside bathi g solution. Effect of f l pin on the short-current and potential across the frog sk n. A. At the rrow f lipin was added to the inside to gi conentration f 50 μM B. Co trol kin half —— short-circuit current (μA/cm²) O—O potential O—O resistance (Kohm m²).

the inside bathing solution of the frog skin results in an activation of the active so transport.

Effect on passive fluxes

To evaluate the effect of filipin on the permeability of the frog skin to substances, the tran of which can be accounted for by passive forces, transepithelial sucrose and chlorid

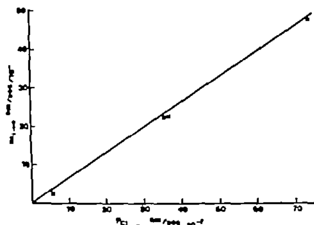


Fig. 3. The sodium outflux plotted against the chloride outflux. The outfluxes of sodium and chloride were measured simultaneously on the same skin after the addition of filipin (30 μM) to the outside bathing medium.

results in the formation of a pathway in the outward facing membrane that is insensitive to amiloride.

Fig. 5, which is representative for 8 expts., shows that the addition of filipin to the inside bathing solution has no effect on the SCC when this was blocked by amiloride. After the outside bathing solutions were replaced by solutions without amiloride, the control skin half showed the normal filipin induced changes in the SCC. Thus the effect of filipin added to the inside bathing solution is not due to a formation of an amiloride insensitive sodium pathway but is the result of an increase in the sodium flux via the amiloride sensitive sodium pathway.

Effect on cell volume

Although addition of filipin to the inside had a very small effect on the transepithelial permeability it remained still an open question whether filipin caused a large increase in the permeability of the inward facing cell membranes. If filipin caused a large increase in the non-specific permeability of the inward facing cell membranes, the cells would swell. To

TABLE V. Effect of amphotericin B on the permeability of the frog skin to chloride and urea. 50 μM amphotericin B in the inside bathing solution.

Period	Number of expts	Control ± S.E. A–B	Amphotericin B ± S.E. B–C	C–D
			cm sec⁻¹ 10⁻⁷	
Urea	5	5.38 ± 0.57	5.39 ± 0.60	5.26 ± 0.62
Chloride	6	5.00 ± 0.61	4.57 ± 0.75	4.93 ± 1.38

In each expt. following 20 min equilibration for the isotopes the permeability across the skin was determined for one, 1 h control period (period A–B). Amphotericin B was then added to the inside bathing medium. After the addition of the amphotericin B solution, the permeability was determined for 2 further 1 h periods (period B–C, C–D). All values are the means ± S.E.

TABLE III Effect of filipin on the permeability of the frog skin to chloride and sodium. 50 μM filipin the outside bathing medium.

Period	Number of expts.	Control ±S.E. A B	Filipin ±S.E. B–C	Filipin ±S.E. C D
		cm sec^{-1} × 10^{-}		
Chloride outflux	8	2.76 ± 0.55	28.1 ± 4.9	43.1 ± 5.3
Chloride influx	9	3.06 ± 0.46	21.3 ± 5.9	32.9 ± 9.9
Sodium outflux	5	1.09 ± 0.41	19.6 ± 5.5	24.6 ± 6.4

In each expt. following 20 min of equilibration for the isotope the permeability across the tissue was determined for one, 1 h period (period A–B). Filipin was then added to the outside bathing medium. After addition of the filipin solution, the permeability was determined for 2 further 1 h periods (periods B–C and C D). All values are the means ± S.E.

B to the inside bathing solution has no effect on the passive chloride and urea flux (Table For both substances in and outflux were measured. Thus the addition of amphotericin and filipin to the outside bathing solution resulted in a significant increase in the passive transepithelial fluxes, for the substances tested, whereas the addition of amphotericin and filipin to the inside bathing solution had no or very small effects on these fluxes.

Action of amiloride

Previous studies have shown that addition of amiloride to the outside bathing solution the frog skin and the toad bladder inhibit the active transepithelial sodium transport (Salako and Smith 1970 Nielsen and Tomlinson 1970 Bentley 1968) This inhibition is believed be caused by an interaction of amiloride with the specific sodium pathway (the sodium channels) in the outward facing membrane (Cuthbert and Shum 1974).

Fig. 4 shows an experiment in which the SCC was blocked by amiloride. The addition filipin to the outside bathing solution caused full recovery of the SCC. In 10 expts. the addition of 50 μM filipin to the outside bathing solution after the addition of amiloride resulted in 20–100% recovery of the SCC. Thus addition of filipin to the outside bathing solution

TABLE IV Effect of filipin on the permeability of the frog skin to chloride and sodium. 50 μM filipin the inside bathing medium

Period	Number of expts	Control ±S.E. A B	Filipin ±S.E. B–C	Filipin ±S.E. C D
		cm sec^{-1} × 10^{-}		
Chloride outflux	7	2.26 ± 0.48	3.04 ± 0.59	3.49 ± 0.75
Chloride influx	7	1.84 ± 0.34	[illegible].38 ± 0.43	2.51 ± 0.44
Sodium outflux	5	1.19 ± 0.22	1.66 ± 0.16	1.71 ± 0.66

In each expt. following 20 min of equilibration for the isotope the permeability across the tissue was determined for one, 1 h period (period A B). Filipin was then added to the inside bathing medium. After the addition of the filipin solution, the permeability was determined for 2 further 1 h periods (periods B–C and C D). All values are the means ± S.E.

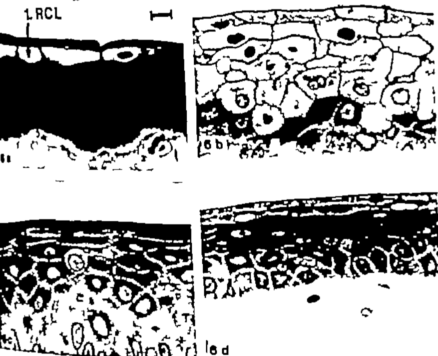


Fig. 6 Light micrographs of frog skin epithelium incubated with 50 µM filipin in the inside- or the outside bathing solution, 1 µm sections stained with toluidine blue. The bar on Fig. 6 corresponds to 10 µm. [illegible] The skin incubated in 1 h with filipin in the outside bathing solution marked swelling of the cells in the 1 RCL observed. 6 b. The skin is incubated in 3 h with filipin in the outside bathing solution. [illegible] swelling of the outermost 4–5 cell layers is observed, and the interspace system is closed by the swollen cells. 6 Control skin. 6 d. The skin is incubated in 3 h with filipin in the inside bathing solution. The cells of the lower cell layers did not show any tendency to swell. Concerning the effect of filipin on the cells in the 1 RCL see text.

[illegible] the possibility expts. were performed in which the skins were incubated with filipin on the inside or on the outside from 10 min to 3 h. After the incubation, the skins were fixed by adding Ringer's solution with OsO_4 to both chamber halves, to give a concentration of 1% OsO_4 in the solutions bathing the skin. Fig. 6 (a, b) (representative for 7 expts.) shows the effect on cell volume of adding 50 µM filipin to the outside bathing solution. After 60 min incubation the volume of all the cells increases (Fig. 6 a) in the outermost layer of the stratum granulosum (the first reacting cell layer 1 RCL). After 3 h incubation the cells of the underlying 1–4 layers are swollen to such an extent that the interspace system between them appears closed (Fig. 6 b). Saladino, Bentely and Trump (1969) have shown that the addition of amphotericin B to the mucosal side of the toad bladder causes a swelling of the cells in the epithelium, and Swelto, Perriol and Lippe (1975) have shown the addition of amphotericin B to the outside of the frog skin results in a swelling of the cells in the 1 RCL. Even after 3 h of incubation with 50 µM filipin in the inside bathing solution the cells in the lower cell layers did not swell (Fig. 6 D representative for 10 expts.). The effect of filipin (inside) on the cells in the 1 RCL was variable. In seven skins it had no effect, in two skins

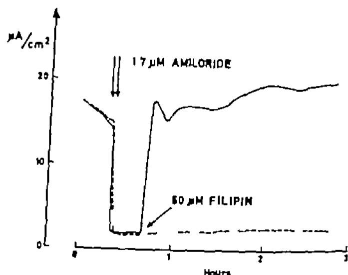


Fig. 4 Effect of filipin (outside) on the SCC of a frog skin where the SCC was blocked by amiloride the arrows amiloride was added to the outside bathing solution of the skin halves to give a concentrat of 1.7 μM At the other arrow filipin (50 μM) was added to the outside of the skin half represented by th solid line.

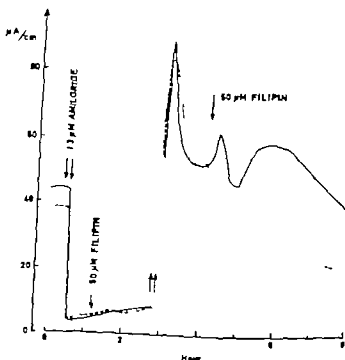


Fig. 5 Effect of filipin (inside) on the SCC of frog skin where the SCC was blocked by amiloride. At the arrows amiloride was added to the outside bathing solution of the skin halves to give a concentration of 1.3 μM At the dotted arrow 50 μM filipin was added to the inside bathing solution of the skinhalf represented by the dotted line. After 3 h of incubation the outside bathing solutions were replaced by fresh Ringer's solution and at the arrow (4 h) 50 μM filipin was added the inside bathing solution of the skin half represented by the solid line.

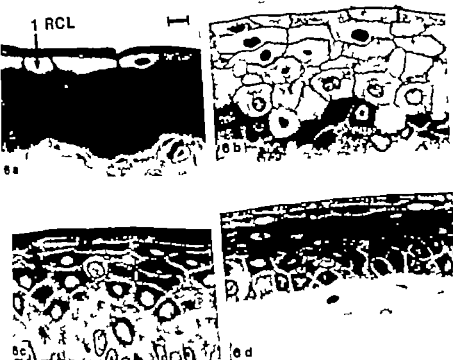


Fig. 6. Light micrographs of frog skin epithelium incubated with 50 μM filipin in the inside- or the outside bathing solution, 1 μm sections stained with toluidine blue. The bar on Fig. 6 corresponds to 10 μm. The skin incubated in 1 h with filipin in the outside bathing solution: marked swelling of the cells in the 1. RCL is observed. 6 b. The skin is incubated in 3 h with filipin in the outside bathing solution. Marked swelling of the outermost 4–5 cell layers is observed, and the interspace system is closed by the swollen cells. 6 c. Control skin. 6 d. The skin is incubated in 3 h with filipin in the inside bathing solution. The cells in the lower cell layers did not show any tendency to swell. Concerning the effect of filipin on the cells in the 1. RCL see text.

test this possibility expts. were performed in which the skins were incubated with filipin on the inside or on the outside from 10 min to 3 h. After the incubation, the skins were fixed by adding Ringer' solution with OsO to both chamber halves, to give a concentration of 1% OsO in the solutions bathing the skin. Fig. 6 (a, b) (representative for 7 expts.) shows the effect on cell volume of adding 50 μM filipin to the outside bathing solution. After 60 min incubation the volume of all the cells increases (Fig. 6 a) in the outermost layer of the stratum granulosum (the first reacting cell layer 1. RCL). After 3 h incubation the cells of the underlaying 1–4 layers are swollen to such an extent that the interspace system between them appears closed (Fig. 6 b). Saladino, Bentely and Trump (1969) have shown that the addition of amphotericin B to the mucosal side of the toad bladder causes a swelling of the cells in the epithelium, and Swetko, Perrini and Lippo (1975) have shown the addition of amphotericin B to the outside of the frog skin results in a swelling of the cells in the 1. RCL. Even after 3 h of incubation with 50 μM filipin in the inside bathing solution the cells in the lower cell layers did not swell (Fig. 6 D representative for 10 expts.). The effect of filipin (inside) on the cells in the 1. RCL was variable. In seven skins it had no effect, in two skins

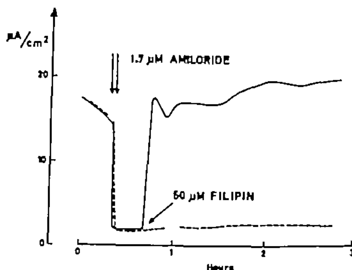


Fig 4 Effect of filipi (utside) n the SCC of a frog skin where the SCC was blocked by amiloride At the arrows amiloride was added to the outside bathi g solution of the skin halves t give a concentration of 1 7 μM At the other arrow f lipin (50 μM) was added to the outside of the skin half represented by the solid line.

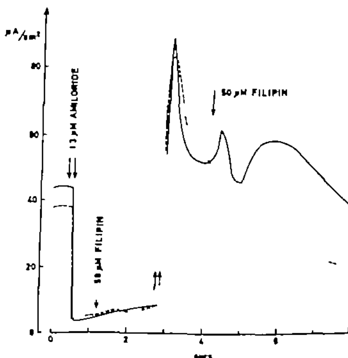


Fig. 5 Effect of filipin (inside) on the SCC of frog ki where the SCC was blocked by miloride. At the arrows amiloride was added to the outside bath g solutions of the skin halves t give concentration of 1.3 μM. At the dotted arrow 50 μM fil pin was added to the inside bathing solution of the skin half represented by the dotted l ne After 3 h of incubation the outside bathing solutions were replaced by fresh Ringer's solution and at the arrow (4 h) 50 μM f lipin was dded the nside bathing solution of the skin half represented by the solid line.

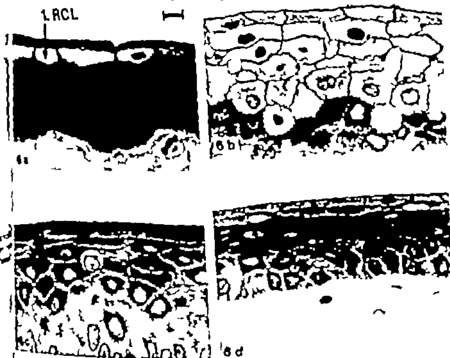


Fig. 6. Light micrographs of frog skin epithelium incubated with 50 μM filipin in the inside or the outside bathing solution, 1 μm sections stained with toluidine blue. The bar on Fig. 6 corresponds to 10 μm. 6 a: The skin incubated for 1 h with filipin in the outside bathing solution marked swelling of the cells of the 1. RCL is observed. 6 b: The skin is incubated for 3 h with filipin in the outside bathing solution. Marked swelling of the outermost 4–5 cell layers is observed, and the interspace system is closed by the swollen cells. 6 c: Control skin. 6 d: The skin is incubated for 3 h with filipin in the inside bathing solution. The cells in the lower cell layers did not show any tendency to swell. Concerning the effect of filipin on the cells of the 1. RCL see text.

Test this possibility expts. were performed in which the skins were incubated with filipin on the inside or on the outside from 10 min to 3 h. After the incubation, the skins were fixed by adding Ringer's solution with OsO₄ to both chamber halves, to give a concentration of 1% OsO₄ in the solutions bathing the skin. Fig. 6 (a, b) (representative for 7 expts.) shows the effect on cell volume of adding 50 μM filipin to the outside bathing solution. After 60 min incubation the volume of all the cells increases (Fig. 6 a) in the outermost layer of the stratum granulosum (the first reacting cell layer 1. RCL). After 3 h incubation the cells of the underlying 1–4 layers are swollen to such an extent that the interspace system between them appears closed (Fig. 6 b). Saladino, Bentely and Trump (1969) have shown that the addition of amphotericin B to the mucosal side of the toad bladder causes a swelling of the cells in the epithelium, and Swelto, Perrini and Lippe (1975) have shown the addition of amphotericin B to the outside of the frog skin results in a swelling of the cells in the 1. RCL. When after 3 h of incubation with 50 μM filipin in the inside bathing solution the cells in the lower cell layers did not swell (Fig. 6 D representative for 10 expts.). The effect of filipin (inside) on the cells in the 1. RCL was variable. In seven skins it had no effect, in two skins

it caused slight swelling—and in one skin a slight shrinkage. Hence addition of filipin to the outside results in an increase in the permeability of the outward facing cell membranes, whereas the addition of filipin to the inside had no or a very small effect on the sodium permeability of the inward facing cell membranes.

Discussion

From the data presented above it appears that the addition of filipin to the outside bathing solution resulted in an increase in the passive transepithelial sodium, chloride, and sucrose fluxes and in a progressiv swelling of the cells in the different cell layers. The addition of filipin to the inside bathing solution however had no effect on the passive transepithelial fluxes and no effect on the volume of the cells. The discussion will be separated in two parts: A, the effect of filipin on the transepithelial fluxes and B the effect of filipin on the progressive swelling of the cells in the different cell layers.

According to the two membrane hypothesis (Koefoed Johnsen and Ussing 1958) the frog skin can be treated as composed of an "outward-facing membrane" which is selectively permeable to sodium ions but impermeable to potassium ions and permeable in a non-selective way to small anions like chloride. The "inward-facing membrane" is permeable to potassium and small anions, but impermeable to free sodium ions. The outward-facing membrane is located just beneath the cornified layer (Ussing and Windhager 1964, Farquhar and Palade 1966). The outward facing membrane consists of the outward-facing cell membranes of the outermost layer of the *s. granulosum* (1. RCL) kept together by tight seals. The inward-facing membrane is identical with the cell membranes limiting the extracellular space throughout the epithelial layer (Fig. 7 A). Thus a solute can permeate across the skin via two types of "pathways": via an extracellular route or via an intracellular route. A solute that permeates via the intracellular route has to cross both an inward and outward-facing cell membrane (Fig. 7 A). A component that only increases the sodium permeability of the outward-facing cell membranes should therefore have no effect on the passive sodium outflux (the inward-facing membranes is impermeable to free sodium), whereas a component which increases the sodium permeability of the inward-facing membrane should increase the passive sodium outflux.

Addition of filipin to the outside results in an increase in the amiloride insensitive sodium transport (Fig. 4), thus filipin increases the sodium permeability of the outward facing cell membrane. Furthermore it has been shown by Nielsen (1971, 1972) that filipin and amphotericin B increase the potassium permeability of the outward facing cell membrane, too. An increase in the sodium and potassium permeability of the outward facing membrane would result in an increase in the cellular sodium concentration and a decrease in the cellular potassium concentration. These changes in the cellular compositions should result in an increased sodium transport. Such an increase was only observed in half the experiments. But if the addition of filipin to the outside bathing solution makes the outward facing membrane so permeable to sodium that the sodium flux into the cells exceeds the capacity of the sodium pump, the cells would undergo colloid-osmotic swelling and eventually burst. The

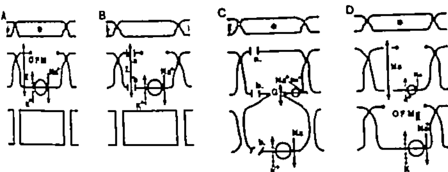


Fig 7 Diagram of the 3 outermost layers of the isolated frog skin. The top layer (the hatched layer) is the corneum The underlying layer is the s. granulosum (1 RCL). The 1 RCL (1 reacting cell layer) i ... permeable to sodium (indicated by the dots) 7 A. OFM, outward facing membrane. I, intracellula ... 7 B and b, disintegrations of the outward (a) and (b) the inward facing cell membranes. 7 C. G, gap junction formed as result of the filipin induced swelling of the 1 RCL. For further information see text 7 D Filipin induced changes in the 1 RCL could result in formation of new outward facing cell membrane (OFM_{II}) in the underlying cell layers. For further information see text.

variable effect of filipin on the SCC could readily be understood if filipin in some skins results in a fast and extensive cytolysis of the cells. The data of Fig. 6 A show that addition of filipin to the outside solution results in a swelling of the cells.

This swelling of the cells would most likely result in a partial disintegration of both the inward and the outward facing cell membranes (Fig. 7 B). A partial disintegration of the inward and outward facing cell membranes would result in a nonspecific increase in the passive transepithelial permeability which could explain the increase in the passive transepithelial fluxes of sucrose, chloride and sodium (Tables II, III and Fig. 3).

Since the addition of filipin to the inside bathing solution has no effect on the sodium outflux, filipin (according to the two membrane hypothesis explained above) has no effect on the sodium permeability of the inward facing cell membrane, and therefore it should have no effect on the cell volume and the passive transepithelial permeability That this interpretation is correct appears from the data of Tables II, IV and Fig. 6 D The addition of filipin to the inside results, however in an increase in the active sodium transport (Fig. 2, Table II). This is probably caused by an indirect effect of filipin on the potassium permeability of the inward facing membrane, mediated by calcium entry (Nielsen 1976).

Many studies show that the presence of sterols in the membrane is a requirement for polyene antibiotic sensitivity (for references see Norman *et al.* 1972). If as suggested by Kruijff and Demel (1974) the filipin induced permeability changes are a result of filipin forming large filipin cholesterol complexes in the membrane, the outward-facing membrane must be assumed to have a high cholesterol content, because filipin *per se* only increases the non specific permeability of the outward-facing membrane; the cholesterol content of the inward-facing membrane must be low

After 1 h of incubation with filipin (outside) the cells of the 1 RCL are swollen and have a necrotic appearance (Fig. 6 A) none the less most of the skins are still able to generate a high SCC, whereas the skins are unable to generate a SCC when nearly all the cells in the

epithelium are swollen. Therefore, presumably *after 1 h of incubation the 1 RCL* consists of cells which are unable to produce a SCC, and the cells in the underlaying cell-layers generate *the SCC* Under these circumstances sodium can diffuse easily from or through the cells *in the 1* RCL to the cells in the underlaying layers. However Voûte and Ussing (1968) have shown that only the 1 RCL swells during short-circuiting. This result, and the kinetic analysis of sodium and lithium movements in the isolated frog skin by Morel and Leblanc (1973) show that the diffusion from cells in one layer to those in the adjacent ones is very slow This implies that in normal frog skin the cells of the 1 RCL are responsible for the major part of the active transepithelial sodium transport That is supported by expts. of Voûte and Ussing (1970) which show that expansion of the interspace system have no effect on the SCC (in these expts the interspaces (apart from the tight seals) were so expanded that the cells only were in contact via the desmosomes) Since filipin *per se* only increases the non specifi permeability of the outward-facing membrane of the 1 RCL, this effect cannot explain the increase in the direct sodium flow from cell to cell in neighbouring layers. The following two models (A and B) can explain the increase in the direct passing of sodium from one cell layer to the next A when the cells in the 1 RCL swell they come in contact with the cells of the underlaying cell layer resulting in the formation of new gap junctions[1] (Fig 7 C) or B, the filipin-induced changes in the 1 RCL could result in the release of a signal which caused a change in the outward-facing membrane of the underlaying cell layer so that this cell layer becomes sensitive to filipin Fig. 7 D (*e.g* these cells might form a new outward-facing sodium selective membrane) This hypothesis can explain how an epithelium with all the cells in the 1 RCL having a marked necrotic appearance still can transport sodium and it can explain the observed progressive swelling of the underlaying cell layers.

Experiments are in progress to investigate whether the filipin induced necrosis of a cell layer results in the formation of gap junctions or results in the formation of a new outward facing cell membrane of the cells facing the necrotic cells

References

ANDERSEN, B and H H USSING, Solvent drag on non electrolytes during osmotic flow through isolated toad skin and its response to antidiuretic hormone. *Acta physiol. scand* 1957 39 228–229

BENTLEY P J Amiloride a potent inhibitor of sodium transport across the toad bladder *J Physiol* (Lond.) 1968. *195* 317 330.

CUTHBERT A. W and W K SHUM, Binding of amiloride to sodium channels in frog skin. *Mol Pharmacol* 1974 *10* 880–891

DE KRUIJFF B and R. A DEMEL, Polyene antibiotic-sterol interactions in membranes of acholeplasma laidlawii cells and lecithin liposomes. *Biochim biophys A* (Amst) 1974. *339* 57 70.

FARQUHAR, M G and G E. PALADE, Adenosine triphosphatase localization in amphibian epidermis *J cell. Biol.* 1966. *30* 359–379

FINN, A. L., Separate effects of sodium and vasopressin on the sodium pump in toad bladder *Amer J Physiol.* 1968 *215* 849–856.

KINSKY S. C., Comparative responses of mammalian erythrocytes and microbial protoplasts to polyene antibiotics and vitamin A. *Arch. Biochem.* 1963 *102* 180–188

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[1] Loewenstein (1973) has shown that junctional passageways (gap junctions) form rapidly where cells

Koefoed-Johnsen, V. and H. H. Ussing, The nature of the frog skin potential. *Acta physiol. scand.* 1958, 42 298–308.

Lichtenstein, N. S. and A. Leaf. Effect of amphotericin B on the permeability of the toad bladder. *J. clin. Invest.* 1965, 44 1328–1342.

Loewenstein, W. R. Cell coupling. In *Transport Mechanisms in Epithelia*. Alfred Benzon Symposium V. eds. H. H. Ussing and N. A. Thorn. Munksgaard, Copenhagen 1973, 20–76.

Morel, F. and G. Leblanc, Kinetics of sodium and lithium accumulation in isolated frog skin epithelium. In *Transport Mechanisms in Epithelia*. Alfred Benzon Symposium V. eds. H. H. Ussing and N. A. Thorn. Munksgaard, Copenhagen 1973, 73–82.

Nielsen, R., Effect of amphotericin B on the frog skin in vitro. Evidence for outward active potassium transport across the epithelium. *Acta physiol. scand.* 1971, 83, 106–114.

Nielsen, R., The effect of polyene antibiotics on the aldosterone induced changes in sodium transport across isolated frog skin. *J. Ster. Biochem.* 1972, 3, 121–128.

Nielsen, R. Effects of aldosterone on frog skin. In *Transport Mechanisms in Epithelia*. Alfred Benzon Symposium V. eds. H. H. Ussing and N. A. Thorn. Munksgaard, Copenhagen 1973, 214–224.

Nielsen, R. Does the polyene antibiotic filipin act as Ca ionophore, when added to the inside of the isolated frog skin. *Acta physiol. scand.* 1976, Suppl. 440, 72.

Nielsen, R. and R. W. S. Tomlinson, The effect of amiloride on sodium transport in the normal and moulting frog skin. *Acta physiol. scand.* 1970, 79, 238–243.

Norman, A. W., R. A. Demel, B. De Kruyff, W. S. M. Geurts van Kessel and L. L. M. van Deenen, Studies on the biological properties of polyene antibiotics: Comparison of other polyenes with filipin in their ability to interact specifically with sterol. *Biochim. biophys. Acta* (Amst.) 1972, 290, 1–14.

Rawlins, F., L. Mateu, F. Fragachan and C. Whittembury. Isolated toad skin epithelium. Transport characteristics. *Pflügers Arch. ges. Physiol.* 1970, 316, 64–80.

Saladino, A. J., P. J. Bentley and B. F. Trump. Ion movements in cell injury. *Amer. J. Pathol.* 1969, 54, 421–466.

Salako, L. A. and A. J. Smith, Changes in sodium pool and kinetics of sodium transport in frog skin produced by amiloride. *Brit. J. Pharmacol.* 1970, 39, 99–109.

Sella, M., M. C. R. Pierini and C. Lippe, Anomalous effects of amphotericin B on the non-electrolyte fluxes through the skin of Rana Esculenta. *Gen. Pharmacol.* 1975, 6, 105–108.

Ussing, H. H. and K. Zerahn, Active transport of sodium as the source of electric current in the short circuited isolated frog skin. *Acta physiol. scand.* 1951, 23, 110–127.

Ussing, H. H. and E. Windhager, Nature of the shunt path and active sodium transport path through frog skin epithelium. *Acta physiol. scand.* 1964, 61, 484–504.

Van Zutphen, H., R. N. Demel, A. W. Norman and L. L. M. Van Deenen, The action of polyene antibiotics on lipid bilayer membranes in the presence of several cations and anions. *Biochim. biophys. Acta* (Amst.) 1971, 241, 310–330.

Voûte, C. L. and H. H. Ussing, Some morphological aspects of active sodium transport. *J. cell Biol.* 1968, 36, 625–638.

Voûte, C. L. and H. H. Ussing, Quantitative relation between hydrostatic pressure gradient, extracellular volume and active sodium transport in the epithelium of the frog skin (R. Temporaria). *Exp. Cell Res.* 1970, 62, 375–383.

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Extracellular Potassium Concentration in Juvenile and Adult Rat Brain Cortex *during* Anoxia

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Abstract

HANSEN A. J *Extracellular potassium concentration in juvenile and adult rat brain cortex during anoxia* Acta physiol scand. 1977 99 412-420

The extracellular K+ concentration, ([K]ₑ), in the brain cortex of rats at different ages was measured by means of K -sensitive microelectrodes. [K]ₑ was between 3 and 5 mM at all ages. Following nitrogen inhalation there was an increase [K]ₑ which exhibited sigmoid pattern in every age group. Firstly there was slow rate of rise which was followed by sudden, steep increase where the [K]ₑ rose to about 70 mM. During the subsequent 5-10 min a plateau value of about 90 mM was reached. The prominent difference between the age groups was the time until the steep rise began. The time to the steep increase in [K]ₑ and the [K]ₑ at the start of the steep increase was inversely related to the age of the animal. A close relation was found between the time to the start of the steep increase and the time to the last gasp during nitrogen breathing (r=0.98). It is suggested that the different resistence to anoxia in young and adult animals is related to differences in the ability to keep near normal potassium gradients across the cells in the brain.

It is well established that young animals survive longer periods of anoxia than adults. It has been shown that the rate of rise in the potassium concentration of the cerebrospinal fluid (CSF) following brain ischemia in rats was correlated with the age of the animal (Hansen 1976 b) Since a high concentration of potassium extracellularly seriously impairs brain function by depolarizing the nerve cells it was thought that the ability to keep extracellular potassium concentration ($[K]_e$) near normal values would reflect the tolerance of the brain to a period of anoxia. Due to the long diffusion distance from the tissue to CSF the CSF potassium values are distorted in relation to tissue values in the unsteady state. In order to circumvent this problem K+ selective microelectrodes were used to measure $[K]_e$ directly in the brain cortex. The aim of the investigation was therefore to examine whether differences in the ability to regulate $[K]_e$ during anoxia in the brain cortex of immature and mature rats might be correlated to the known differences in tolerance of anoxia. A preliminary report has been given (Hansen 1976 a)

Methods

Sprague-Dawley rats were studied. Their ages and numbers were as follows 4 days, 4 animals 7 days, 6 animals 12 days, 5 animals, 16 days, 6 animals 24 days, 4 animals and adults, 7 animals. The rats were

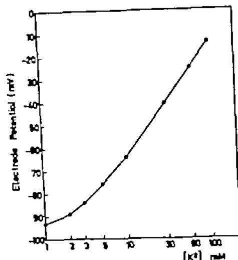


Fig. 1 Calibration curve of a double-barreled K ion-sensitive microelectrode obtained in solutions of different concentrations of KCl with NaCl added to maintain constant ion strength.

[illegible] with pentobarbital administered i.p. (20–30 mg/kg in young rats and 50 mg/kg in the adults), tracheotomized and placed on a heated table. The body temperature was maintained at 37°C by a servo-controlled heating system. The rats breathed spontaneously and the respiration rate was measured with a thermistor inserted in the tracheal tube and the ECG was recorded from needles in the front legs. Blood pressure was monitored only in the adults, via a catheter in the femoral artery. The head of the adults was secured in a headholder. Since the heads of the juvenile rats are not sufficiently ossified to withstand the pressure from the earpins, their heads were immobilized between two pointed forks. Thereafter a trephine opening 3–4 mm in diameter was made in the left parietal bone. In the adults the dura was opened with a sharp needle, but in the other animals it was left intact. The respiratory rate, ECG and the blood pressure were recorded on a 4-channel Beckman recorder. The animals were made anoxic by letting them breathe pure nitrogen.

K⁺-sensitive microelectrodes

The electrodes were made as described by Zeuthen *et al.* (1974). Double-barrelled micropipettes were pulled from two aligned capillary tubes, which were offset 1 cm along the long axis. This produced pipettes with one long and one short barrel. The tip diameter was about 1 μm as determined by light microscopy. The long barrel was siliconized by exposing the open backend to the vapor of dichlorodimethyl-silane for [illegible]. The pipette was then dried in an oven at 100°C for 1 h. The shank of the siliconized barrel was filled from behind with potassium exchanger (Corning 477317) using a thin capillary tube. Usually the exchanger would fill the tip immediately. The rest of the long barrel and the short barrel was then filled with 150 mM KCl. Air bubbles [illegible] the pipette were removed using the principle of Zeuthen (1971). A 50 μm heated wire was applied to the outside of the electrode at the air–water or air–ion exchanger interface furthest away from the tip. The water or exchanger were thereby distilled towards the tip, while the bubble moved away from the tip. Since the exchanger has a higher boiling temperature than a 150 mM KCl solution, bubbles in the exchanger were removed before filling the reference barrel.

The potential arising between the barrels is a measure of the K⁺ activity at the tip. The electrodes were calibrated in solutions containing different concentrations of KCl with NaCl added to ensure constant ion strength. The calibration curve derived in this way was used to convert the measured K⁺ activity to K⁺ concentration (Fig. 1). The potential of the reference barrel varied in most electrodes less than 3 mV in the different test solutions. Electrodes with higher values were discarded. The electrodes typically showed 45–48 mV change for a 10-fold change in K⁺-concentration. The resistance of the potassium barrel varied between 10^9 and 10^{10} Ω. Ag/AgCl connections led from both barrels via shielded cables to a Keithley differential electrometer 604 (input impedance 10^{14} Ω). The potential between the reference barrel and the ground was measured by recording the negative gun output with respect to a grounded glass tube filled with 150 mM KCl solidified in agar. The K⁺ activity and the tissue potential were recorded in individual

TABLE I Extracellular K concentration in brain cortex of rats t different ages. The alues, expressed i mM are means ± S.D

	Age (days)					
	4	7	12	16	24	Adult
$[K]_e$	4 6±1 3	2.9±0.4	3 4±0.6	3 1±0.5	4.3±0.4	3.0+0 5

channels on a pen writer The response time of the system was less than 1/2 s. The animal as grounded t the agar filled glass tube buried in a neck muscle.

The brain electrode was inserted with a micro-manipulator controlled by a step motor In all animals th K activity was measured at a depth of 0.5 mm. The K activity recorded at this depth as constant fo at least 5 min before nitrogen was administered to the animal. The electrodes were cal brated before an just after the expts. If there were significant differences (>2 mV) between the two calibrations the result were discarded

Results

$[K]_e$ resting level

The base-line value of $[K]_e$ in the brain cortex of rats at different ages are shown in Table I At all ages the resting values were between 2.9–4 6 mM

$[K]_e$ in the brain following anoxia

The rise following nitrogen inhalation exhibited the same pattern at all ages (Fig. 2). Firstly there was a slow rate of rise, which was followed by a sudden steep increase, where the $[K]_e$ rose to about 70 mM Hereafter it slowly increased until it reached a final level of about 90 mM after 10 min The prominent difference in the response between the age groups was the time until the steep increase began. In the 4-day-old rat it took about 20 min to reach the steep increase, while in the adult it took only 1 5 min. In fact, the time delay was inversely related to the age of the animal (Fig. 3). In the 4 7 and 12-day-old rat the steep increase began at a $[K]_e$ of about 25 mM but in rats older than 12 days it began at about 10 mM

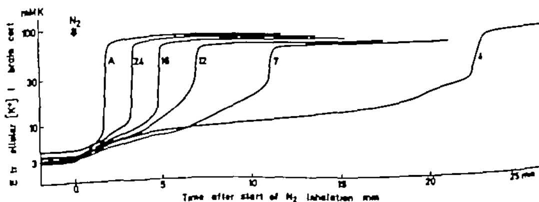


Fig. 2. $[K]_e$ in the brain cortex of rats t different ges following exposure to nitrogen. The number indicates the age (days) of the animal. A stands for adult. The figure show representative experiments.

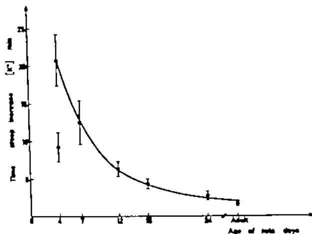


Fig. 3 The time to the start of the steep increase in $[K]_e$ in the brain cortex following nitrogen inhalation in rats at different ages (●) and following removal of the heart in 4-day-old rats (○). 1 S.D. is indicated by vertical bars.

(Fig. 4). The slope of the steepest part of the K curve was related to the age of the animal (Fig. 5).

The steep increase in $[K]_e$ was at all ages accompanied by a negative shift in the tissue potential, called anoxic depression (Leão 1947, Bureš and Burešová 1957).

$[K^+]_e$ and last gasp following anoxia

To investigate whether there was a relationship between the time to the last breath following anoxia and the time to the steep increase in $[K]_e$ a plot was made and the correlation coefficient was calculated (Fig. 6). There was a close relationship between the two parameters (r = 0.98).

$[K]_e$ changes following brain ischemia

To examine the significance of the circulation on the $[K]_e$ in the brain during anoxia, the heart was removed in four 4-day-old rats. Within 2 min the rat was mounted in the head-

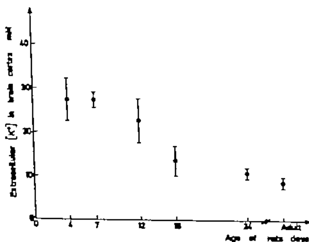


Fig. 4. $[K]_e$ at the start of the steep increase in $[K]_e$ in brain cortex of rats at different ages following nitrogen inhalation. 1 S.D. indicated by vertical bars.

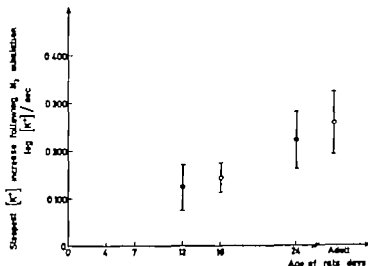


Fig. 5. The slope of the steepest increase in $[K]_e$ in the brain cortex of rats at different ages following nitrogen inhalation. ±S.D. is indicated by vertical bars when exceeding the symbols

holder and the electrode inserted in the brain cortex. The $[K]_e$ increased faster in these animals and the steep increase was reached in 9–10 min instead of 20 min in similar rats with intact cardiovascular system (Fig. 3) but the [K] at the beginning of the steep increase was not changed. In 4 adults the heart was arrested by injection of concentrated $MgCl_2$ through the femoral vein. Previous expts. with Evans blue added to the MgCl solution showed no visible staining of the brain during this procedure. This method of arresting the circulation did not affect the course of events significantly.

Reversibility of the anoxic $[K]_e$ increase

In other expts. the nitrogen supply was disconnected in adult rats just after the steep increase in $[K]_e$ had occurred, whereafter the animals were allowed to breathe air. In most of the

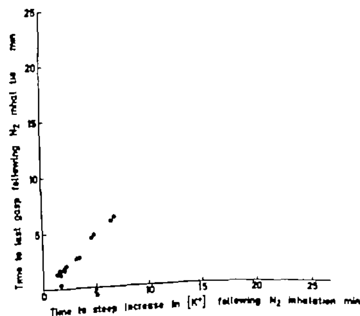


Fig. 6. The correlation between the time to the last gasp and the time to the start of the steep increase in $[K]_e$ in brain cortex of juvenile and adult rats. r = 0.975

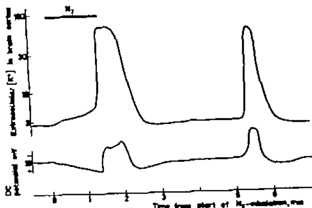


Fig. 7 Changes in $[K]_e$ and the extracellular potential in the brain cortex of an adult rat during and after nitrogen inhalation. Note the spontaneous, transient increase in $[K]_e$ and the change in the steady potential characteristic of Leão's spreading depression.

was $[K]_e$ in the brain returned to preanoxic levels (Fig. 7). This procedure was sometimes followed by an increase in $[K]_e$ characteristic of Leão's spreading depression (Vyskočil *et al.* 1972). A prerequisite for the return of the $[K]_e$ was that the cardiovascular system responded to the air breathing by an increase in blood pressure. Otherwise the steep increase was irreversible.

Discussion

$[K]_e$ level following anoxia. It was shown in the present investigation that $[K]_e$ in the brain cortex was 3-5 mM in juvenile and adult rats. This is in accordance with the results obtained by Mutani *et al.* (1974) who studied immature rabbits.

Following nitrogen inhalation a slow rise in $[K]_e$ was found which was later followed by a steep increase reaching a final $[K]_e$ level of about 90 mM (Fig. 2). The fact that $[K]_e$ rose in a sigmoid fashion following anoxia has no simple explanation. The cells in the brain are liable to lose their K^+ following anoxia since the high intracellular K^+ concentration is maintained by energy dependent processes. The virtual impermeability of the blood-brain barrier to K^+ (Crone and Thompson 1970, Hansen and Lund-Andersen 1976) prevents a rapid escape of the K^+ to the blood and thus an increased $[K]_e$ is maintained.

The initial increase in $[K]_e$ occurred at a relatively slow rate and may be caused by a passive leak of K^+ not counteracted stoichiometrically by the ion pumps in the cell membrane, due to a reduced supply of ATP. A reduction in the extracellular space would also increase the $[K]_e$, but the changes observed from conductivity measurements (Van Harreveld 1977) are too small to account for the observed changes. The duration of the initial rise in $[K]_e$ became steadily shorter during ontogenesis (Fig. 2). This might be explained by a faster depletion of ATP in the brain cortex due to the increase in cerebral metabolic rate with age (Duffy *et al.* 1975) in combination with a decrease in the ability of the heart to withstand anoxia (Dawes *et al.* 1959).

Bureŝová *et al.* (1957) studied in isolated heads of adult rats the time to the negative shift in tissue potential, which is a phenomenon that accompanies the steep increase in $[K]_e$,

They found that the time was prolonged by decreasing the cerebral metabolic rate wi barbiturates and hypothermia. Glucose administered i p increases the glucose stores in th brain cortex (Ljunggren *et al* 1974) Anaerobic glycolysis might hereby provide ATP fo a longer time during anoxia. Hansen (unpublished) found that pretreatment with glucos in the adult rat delays the occurrence of the steep increase in $[K]_e$ following brain ischemia. Thus, the duration of the initial increase in $[K]_e$ seems to be dependent on the ability of th brain to maintain sufficient ATP levels, and the transition to the steep increase may b related to a low ATP level in the brain, but no experimental data are available. At the tim of the negative shift in tissue potential there is a decrease in the extracellular space of th brain cortex (Van Harreveld 1972) due to cellular uptake of Na, Cl and water but since th decrease in space computed from conductivity measurements only amounts to about 50 it cannot solely explain the magnitude of the steep rise in $[K]_e$. Another possibility is a increase in the K^+ permeability (P_K) of the cell membrane. The change in P_K could be in duced by an increase in $[K]_e$ since this would lead to a depolarization of the cells and thereb to an increase in the P_K of the neurons (Hodgkin and Huxley 1952). It is well known tha application of KCl on the cerebral cortex elicits a spreading depression (Leão 1944), whic involves a rapid rise in $[K]_e$ and a concomitant negative shift in the tissue potential simila to the events during anoxia (Fig. 7) However in rats younger than 12 days the application o KCl did not elicit a spreading depression, but only a local, negative potential shift (Dex and Eidelberg 1967) The present study has shown that a negative shift in tissue potentia at all ages accompanies a rapid rise in $[K]_e$. Lothmann *et al* (1975) found that if the $[K]_e$ exceeded 12 mM following electrical stimulation a spreading depression invariably followed. Assuming that the mechanism is the same for the steep increase in [K] during a spreading depression and following anoxia, these results suggest that the initial increase in $[K]_e$ during anoxia is responsible for eliciting the subsequent increase in $[K]_e$.

Also a release of Ca^{++} from the mitochondria due to a reduced level of ATP (Krnjević 1975) and cellular swelling (Hendil and Hoffmann 1974) which accompanies anoxia, could induce a rise in P_K Cellular swelling is not a likely possibility since shrinkage of the cells in the brain with a hypertonic solution before anoxia did not affect the time to the steep increase in [K] following anoxia (Hansen unpublished) However increase in intracellular Ca^{++} might be a possible candidate for eliciting the assumed increase in P_K following anoxia, but data on this matter are not available

From Fig. 2 it appears that the $[K]_e$ at the beginning of the steep rise in $[K]_e$ decreases with age The reason for this is unclear but a possibility might be that the immature neuron is less permeable to K than that of the adult A sufficient depolarization for a rise in P_K could thus only be reached with a higher $[K]_e$. Supporting this idea is the study of Mutani *et al* (1974) who found that immature rabbit brain cortex could generate epileptiform activity with a $[K]_e$ of at least 20 mM

The rate of rise of $[K]_e$ during the steep increase became steadily faster during ontogenesis (Fig. 5) An increase in surface-volume ratio of the neurons (Eayrs and Goodhead 1959) and a decrease in the extracellular space (Vernadakis and Woodbury 1962) could explain this finding.

In the discussion I have focused upon the neurons as the source of K during the steep

[illegible] in $[K]_e$. However it is not known if this is correct or if the glia are involved. In the future we shall try to investigate this problem.

$[K]_e$ level and anoxic survival. The gradual decrease in the tolerance to anoxia which occurs during ontogenesis (Fazekas *et al.* 1941) may be related to the ability to maintain the normal $[K]_e$ in the brain under anoxic stress. Several studies corroborate such an idea. Adolph (1969) found survival times for young and adult rats exposed to nitrogen similar to the time intervals for the appearance of the steep increase in $[K]_e$ reported in this study. Duffy *et al.* (1975) found that 7-day-old rats survived nine times longer than adults, while this study has shown that the time to reach the steep increase in $[K]_e$ in 7-day-old rat was eight times longer than in the adult. The criteria for survival in the above mentioned studies was the ability of the animals to recover spontaneously after anoxia. Other studies have shown that the survival after anoxia parallels persistent gasp reflex and have therefore used the duration of gasping during anoxia as the length of anoxic survival (Selle and Witten 1941). In the present investigation a correlation coefficient of 0.98 was found between the time to the last gasp and the time to the steep increase in $[K]_e$.

The steep increase in $[K]_e$ following anoxia is a reversible phenomenon if the cardiovascular system is still functioning. A close relationship has been found between cardiac content of carbohydrate and the survival of rats at different age following anoxia (Stafford and Weatherall 1960). In this study the time until the steep increase in 4-day-old rats was halved by stopping the heart in comparison with animals inhaling nitrogen, while no difference was observed in the adult. It should therefore be emphasized that the reported change in the ability to regulate $[K]_e$ of the brain cortex following anoxia which takes place during ontogenesis is due to different tolerance of both the brain and the cardiovascular system to anoxia. The evidence summarized above supports the idea that the different resistance to anoxia in young and adult animals is related to the ability to keep near normal K gradients across the cells in the brain.

References

Adolph, E. F. Regulations during survival without oxygen in infant mammals. *Resp. Physiol.* 1969 *7* 356–368.

[illegible], O., J. Bureš and O. Burešová, Die Wirkung des Chlorpromazins und der Glykämie auf das [illegible] kontrollierte Überleben der Hirnrinde bei verschiedenen Körpertemperaturen. [illegible] *Arch. exp. Path. Pharmak.* 1957 *231* 550–561.

Bureš, J. and O. Burešová, Die anoxische Terminaldepolarisation als Indicator der Vulnerabilität der Grosshirnrinde bei Anoxie und Ischämie. *Pflügers Arch. ges. Physiol.* 1957 *264* 325–334.

Crone, C. and A. M. Thompson, Permeability of brain capillaries. I. *Capillary Permeability. Alfred Benzon Symposium II.* Eds. Crone, C. and N. A. Lassen. Munksgaard, Copenhagen. 1970. 447–453.

Dawes, G. S., J. C. Mott and H. J. Shelley. The importance of cardiac glycogen for the maintenance of life in foetal lambs and newborn animals during anoxia. *J. Physiol.* (Lond.) 1959 *146* 516–538.

Deza, L. and E. Eidelberg, Development of cortical electrical activity in the rat. *Exp. Neurol.* 1967 *17* 425–438.

Duffy, T. E., S. J. Kohle and R. C. Vannucci, Carbohydrate and energy metabolism in perinatal rat brain: relation to survival in anoxia. *J. Neurochem.* 1975 *24* 271–276.

Eayrs, J. T. and B. Goodhead, Postnatal development of the cerebral cortex in the rat. *J. Anat.* (Lond.) 1959 *93*, 385–402.

Fazekas, J. F., F. A. D. Alexander and H. E. Himwich, Tolerance of the newborn to anoxia. *Amer. J. Physiol.* [illegible]

HANSEN A. J Extracellular potassium activity in juvenile and adult rat brain cortex during anoxia. *Acta physiol scand* 1976 *96* 42A–43A.

HANSEN A. J The potassium concentration in cerebrospinal fluid in young and adult rats following complete brain ischemia. Effects of pretreatment with hypoxia. *Acta physiol. scand.* 1976. *97* 519–522.

HANSEN A. J and H. LUND-ANDERSEN, K -permeability of the blood-brain barrier investigated by aid K -selective microelectrodes. *Act physiol scand.* suppl. 1976. *440* 83

HENDIL, K. B and E. K. HOFFMANN Cell volume regulation in *Ehrlich ascites* tumor cells. *J cell Physiol* 1974 *84* 115–126.

HODGKIN A. L. and A. F HUXLEY A quantitative description of membrane current and its application t conduction and excitation in nerve. *J Physiol.* (Lond.) 1952. *117* 500–544.

KRNJEVIĆ, K. Coupling of neuronal metabolism and electrical activity In *Brain Work. Alfred Benzon Symposium VIII* Eds. Ingvar D H and N A. Lassen. Munksgaard, Copenhagen. 1975 65–78.

LEAO, A A. P Spreading depression of activity in cerebral cortex. *J Neurophysiol* 1944. *7* 359–390

LEAO A. A. P Further bservations on the spreading depression of activity in the cerebral cortex. *Neurophysiol.* 1947 *10* 409–419

LJUNGGREN, B K. NORBERG and B K. SIESJÖ, Influence of tissue acidosis upon restitution of brain energy metabolism following total ischemia. *Brain Res.* 1974 *77* 173–186.

LOTHMAN E., J LAMANNA, G CO DINGLEY M. ROSENTHAL and G SOMJEN Responses of electrical potential, potassium levels, and oxidative metabolic activity of the cerebral neocortex of cats. *Brain. Res* 197 *88* 15–36.

MUTANI R., K. J FUTAMACHI and D A. PRINCE, Potassium activity in immature cortex. *Brain Res* 197 *75* 7–39

SELLE, W A and T A. WITTEN Survival of the respiratory (gasping) mechanism in young animals subjected to anoxia. *Proc Soc. xp Biol.* (N Y) 1941 *47* 495–497

STAFFORD A. and J A C. WEATHERALL, The survival of young rats in nitrogen. *J Physiol.* (Lond.) 19 *153* 457–472.

VAN HARREVELD A., The extracellular space in the vertebrate central nervous system. In *The Structure and Function of Nervous Tissue vol. IV* Ed. Bo rne, G H Academic Press. New York. 1972. 477–51

VERNADAKIS, A. nd D M WOODBURY Electrolyte and amino acid changes in rat brain during maturation. *Amer J Physiol.* 1962. *203* 748–752.

VYSKOČIL, F N Kriz and J BUREŠ Potassi m-selective microelectrodes used for measuring the extracellular brain potassium during spread g depression and anoxic depolarization in rats. *Brain Res.* 197 *39* 255–259

ZEUTHEN T A method t f fl glass microelectrodes by local heating. *Acta physiol. scand.* 1971 *81* 141 14

ZEUTHEN, T R C HIAM nd I A. SILVER, Microelectrode recording of ion activity in brain In *Ion-Selective Microelectrodes.* Eds. Berman, H J and N C Hebert Plenum Press, New York. 1974. 145–156.

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Distribution of Coronary Blood Flow in the Left Ventricular Wall of Dogs Evaluated by the Uptake of Xe-133

By

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Abstract

H Distribution of coronary blood flow in the left ventricular wall of dogs evaluated [illegible] uptake of Xe-133 Acta physiol. scand. 1977 99 421–431

[illegible]ution of coronary blood flow was estimated in anaesthetized dogs by counting the activity in [illegible] of the left ventricular free wall immediately after bolus injection of Xe-133 into the aorta [illegible]fferences in the uptake of isotope were observed between the apex and the base of the heart, [illegible] supplied by the anterior descending and circumflex branches of the left coronary artery or [illegible] endo- and epicardial halves of the wall. In most experiments bolus injection of the isotope [illegible] coronary artery was followed by difference in activity between areas supplied by the left [illegible]scending and left circumflex branches. This indicated inadequate mixing of blood and isotope [illegible] stem of the artery. The uneven distribution did not result in differences between the epi- and [illegible] activity concentrations. The results from one normal, anaesthetized dog in which tissue activities [illegible]ured after constant rate infusion of Xe-133 into the left coronary artery for 8 min were in ac[illegible] with the general assumption of equal epi- and endocardial volumes of distribution (values of λ).

[illegible]on of local blood flow from the rate of disappearance of intramyocardial deposits [illegible]31 (Kirk and Honig 1964) and Xe-133 (Harley Harper and Estes Jr 1966, Brandi, [illegible] McGregor 1968) have shown a higher tissue flow in the epi- than the endocardial [illegible] the left ventricular wall of anaesthetized dogs. Myocardial uptake of continuously [illegible] Rb-86 (Love and Burch 1957 Moir and DeBra 1967 Mathes and Rival 1971) and [illegible]tipyrine (Griggs and Nakamura 1968) have indicated a flow (vol time weight⁻¹) [illegible]docardial half of the ventricular wall equal to or higher than that of the epicardial

[illegible]o previous papers (Andersen, Bagger and Gstzsche 1969 Bagger 1972) the two [illegible] ere estimated employing the same isotope Wash-out of Xe 133 after local in[illegible]nto the left ventricular wall showed higher epi- than endocardial flow while myocar[illegible]ake immediately after injection of xenon into the left ventricular cavity was higher [illegible]ndo- than the [illegible] tissue blocks. This implied that conflicting results may

arise from differences in the way of administration of a particular tracer Furthermore the results (Bagger 1972) indicated that, when injected into the left ventricular cavity Xe-1 is able to cross the endocardial surface like Rb-86 (Moir and DeBra 1965 Myers and Hon 1966)

The distribution of radioactive microspheres with a diameter larger than 15 μm tend to exaggerate flow differences in the left ventricular wall of dog hearts (Yipintsoi *et al* 1973 However smaller microspheres (7–15 μm) injected into the left atrium of normal anesthetize dogs in some reports show 10–40% higher endo- than epicardial flow (Fortuin *et al.* 197 Flameng *et al* 1975 Waltier *et al* 1975) while in others reveal a uniform flow (Becke Fortuin and Pitt 1971 Buckberg *et al* 1972 and 1975 Fixler Saunders and Sugg 1974).

In the previous report (Bagger 1972) adequate mixing of blood and tracer was obtaine after bolus injection of Xe 133 into the left ventricular cavity but the use of tissue activit as an indicator of regional blood flow in the left ventricular wall was disturbed by the uptak of isotope across the endocardial surface. When Xe 133 is injected into the aortic root o into the left coronary artery transendocardial uptake of isotope is avoided If adequat mixing of xenon and blood can be obtained by these routes of injection the epi- and endo cardial distribution of activity might be an indicator of flow distribution. The present stud was performed in order to see if adequate mixing of isotope and blood is obtained afte bolus injection of Xe 133 into the aortic root and into the left coronary artery Furthermor the epi and endocardial distribution of activity was measured in order to discuss its relation to flow to these regions. Xe 133 was used in the present experiments for direct comparison of the results to those previously obtained (Bagger 1972)

The fractional volumes of distribution for xenon and other diffusible tracers used fo myocardial blood flow measurements are generally assumed to be identical in the epi- an endocardial part of the ventricular wall (*e.g* Brandi, Fam and McGregor 1968) This as sumption was controlled in one normal anesthetized dog in which tissue activities were measured after constant rate infusion of the isotope into the left coronary artery for 8 min

Methods

15 mongrel dogs of both sexes (19–40 kg) were used for the expts. In 7 dogs (group 1) the uptak of Xe-133 was measured after inject on f the isotope into the aortic root In 7 other dogs (group 2) and also in the d g used for constant rate infusi n, xenon was injected int the left coronary artery

Four dogs in each of the two groups were premedicated w th a subcutaneous injection of methadone 20 mg, tropine 0.5 mg and 2 propyl-10-(dimethylamino-propyl)-flutiazin (Combelene®) 0.5 mg. 30 min later they were anesthetized with thiomebumal sod m (Leopental®) 8 mg/kg v Additional doses of the barbiturate (50–100 mg) were given as required. The other d gs were nesthetized ith thiomebumal sodium (Leopental®) or pentobarbital (Nembutal) 25 mg/kg and similarly supplied with additional 50–100 mg doses. Premedication was used in order t reduce the dose of barbiturate, thereby diminishing the negative inotropic effect on the heart As the result from pre- and non-premedicated dogs were not different, they are not kept separated this report

The dogs were ventilated with tmospheric air through endotracheal tube by means of an Engstrom ventilator Additional oxygen was supplied t two dogs group 2 in which there was low $P\ O_2$ but normal $P\ CO_2$ after thoracotomy This was probably due t telectasis which might develop after opening of the thorax.

A Ducor® left coronary artery catheter was introduced through the right femoral artery and the tip placed temporarily in the descending aorta. A polyethylene catheter was introduced through the left femoral

[illegible] I. Hemodynamic and blood gas parameters of the 14 dogs, measured just before injection of Xe-133. The figures are mean values ± S.D. N = number of dogs.

	Aortic pressure, mmHg		Heart rate beats min⁻¹	P_{O_2} mmHg	P_{CO_2} mmHg	pH
	systolic	diastolic				
[illegible] root injections	133 ± 27 N = 7	102 ± 23 N = 7	144 ± 45 N = 7	77 ± 16 N = 7	35 ± 5 N = 7	7.47 ± 0.08 N = 7
[illegible]ary artery injections	138 ± 12 N = 7	107 ± 13 N = 7	154 ± 13 N = 7	73 ± 14 N = 5	31 ± 6 N = 7	7.40 ± 0.05 N = 7

[illegible] and the tip placed in the descending aorta to allow continuous observation of pressure together with [illegible] recording of the ECG. Thoracotomy was performed on the left side and the pericardium was [illegible]. In the dogs of group 1, the tip of the Ducor catheter was positioned just above the aortic [illegible] [illegible] pointing at the region of the left coronary artery. In group 2 and in the dog exposed to continuous [illegible] of xenon, the tip of the catheter was placed just within the left coronary artery. In all experiments [illegible] position of the catheter was confirmed under fluoroscopy by injection of radiopaque material. 20–30 [illegible] after this injection the oxygen and carbon dioxide tensions and the pH of arterial blood were measured. [illegible] dissolved in saline was then injected as a bolus through the Ducor catheter and the heart immediately [illegible].

[illegible]–6.1 mCi and 0.05–0.14 mCi Xe-133 were injected in group 1 and 2 respectively. During the constant [illegible] 8.6 mCi in 2.9 ml saline was injected per min for 8.4 min, during which period the activity was [illegible] externally by a scintillation detector and a ratemeter.

After excision the left ventricular cavity was flushed with water through the left atrium. This was to [illegible] the blood from the endocardial surface. The heart was then frozen in a mixture of dry ice and [illegible] (−75°C). The apex was removed and the heart was sliced at right angles to the long axis. [illegible] each slice the left ventricular free wall was divided into blocks. Each block was further subdivided [illegible] epi- and endocardial halves and placed in previously weighed, cooled, closed tubes. *In the experiment* [illegible] constant infusion of xenon, the blocks of one slice were divided straightly as described above, while [illegible] blocks of the two other slices were divided in epi- and endocardial halves after removal of the epi- [illegible] with the major vessels. The activity was counted in a well-type scintillation detector with a window [illegible] around the 81 keV peak and expressed as cps/100 mg of tissue.

Results

[illegible]he hemodynamic parameters, blood gas values and pH just before injection of xenon, are [illegible]own in Table I. The mean values of the two groups are not significantly different. In the [illegible] with *left* coronary artery injection the arterial oxygen tensions of the two dogs sup- [illegible]ied with additional oxygen were 164 and 188 mmHg. These figures are not included in the [illegible]ues of the table.

The time interval between bolus injection and heart excision was 14.3 s ± 2.5 (S.D.) and 13 s ± 6.6 (S.D.) *in group 1* and 2 respectively, the longest interval being 26 s. The interval [illegible] excision and freezing of the heart was 2.42–3.07 min, measured in 3 dogs of [illegible] 1. The interval between freezing and placing of the last tissue in its tube was less [illegible] 120 min.

A representative result of the *distribution* of Xe 133 *in* the epi- and endocardial halves of the left ventricular wall after injection of the isotope into the aortic root is shown in Fig. 1. The activity concentrations (cps/100 mg of tissue) of the tissue blocks of each slice are [illegible] in the direction from the left anterior descending branch towards the circumflex

arise from differences in the way of administration of a particular tracer Furthermore the results (Bagger 1972) indicated that, when injected into the left ventricular cavity Xe 133 is able to cross the endocardial surface like Rb-86 (Moir and DeBra 1965 Myers and Hon 1966).

The distribution of radioactive microspheres with a diameter larger than 15 μm tend to exaggerate flow differences in the left ventricular wall of dog hearts (Yipintsoi et al. 1973) However smaller microspheres (7–15 μm) injected into the left atrium of normal anesthetized dogs in some reports show 10–40% higher endo- than epicardial flow (Fortuin et al 197 Flameng et al 1975 Waltier et al 1975) while in others reveal a uniform flow (Becker Fortuin and Pitt 1971 Buckberg et al 1972 and 1975 Fixler Saunders and Sugg 1974).

In the previous report (Bagger 1972) adequate mixing of blood and tracer was obtained after bolus injection of Xe 133 into the left ventricular cavity but the use of tissue activity as an indicator of regional blood flow in the left ventricular wall was disturbed by the uptake of isotope across the endocardial surface. When Xe 133 is injected into the aortic root or into the left coronary artery transendocardial uptake of isotope is avoided. If adequate mixing of xenon and blood can be obtained by these routes of injection the epi- and endocardial distribution of activity might be an indicator of flow distribution The present study was performed in order to see if adequate mixing of isotope and blood is obtained after bolus injection of Xe 133 into the aortic root and into the left coronary artery Furthermore the epi- and endocardial distribution of activity was measured in order to discuss its relation to flow to these regions. Xe 133 was used in the present experiments for direct comparison of the results to those previously obtained (Bagger 1972)

The fractional volumes of distribution for xenon and other diffusible tracers used for myocardial blood flow measurements are generally assumed to be identical in the epi- and endocardial part of the ventricular wall (e g Brandi Fam and McGregor 1968) This assumption was controll d in one normal anesthetized dog in which tissue activities were measured after constant rate infusion of the isotope into the left coronary artery for 8 min

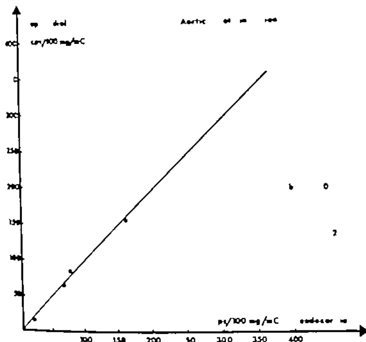
Methods

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Four dogs in each of the two groups were premedicated with a subcutaneous injection of methadone 20 mg, atropine 0.5 mg and 2-propyl-10-(d methylam no-propyl)-flutazin (Combelene®) 0.5 mg. 30 min later they were anesthetized with thiomebumal sodium (Leopental®) 8 mg/kg Additional doses of the barbiturate (50–100 mg) were given as required. The other dogs were anesthetized w th thiomebumal sodium (Leopental®) or pentobarbital (Nembutal®) 25 mg/kg and similarly supplied with additional 50–100 mg doses. Premedication was used in order to reduce the dose of barbiturate, thereby diminishing the negative inotropic effect on the heart. As the result from pre- nd non-premedicated dogs ere not different, they are not kept separated n this report

The dogs were ventilated with tmospheric air through an endotracheal t be by means of an Engström ventilator Additional oxygen was supplied t two dogs group 2 in which there was a low P O₂ but normal P CO_2 after thoracotomy This was probably due t telectasis which might develop after opening of the thorax.

A Ducor® left coronary artery catheter was introduced through the right femoral artery and the tip placed temporarily in the descend ng aorta. A polyethylene catheter was introduced through the left femoral



Comparison of mean epi- and endocardial activity (cpm/100 mg/mCi injected) of the left ventricular 17 dogs after aortic bolus injection.

is not different from unity p 0.50), the correlation coefficient 0.98 and the inter (y 0) 5.6 ± 19.5 (S.D.). The mean ratio of epi- to endocardial activity concentrations 1.98 ± 0.03 (S.E.). Again no correlation was found between the ratio of epi- to endocardial activity concentrations and the time taken to excise the heart.

constant infusion of Xe 133 into the left coronary artery of one dog a slight increase activity recorded by external counting was still observed during the last part of the in- period. In this dog the ratio of epi- to endocardial activity concentrations for all blocks of the left ventricular free wall was 1.02 ± 0.16 (S.D.), n = 34. The slice with epicardium showed a ratio of 1.06 ± 0.13 (S.D.), n = 13 while the ratio for the two slices without epicardium was 1.00 ± 0.18 (S.D.), n = 21. These mean values are not significantly different (p 0.30).

Discussion

the group of dogs subjected to aortic root injection no differences were observed in concentrations of xenon between the apex and the base of the heart or between areas supplied the left anterior descending and circumflex branches of the left coronary artery. These data are in accord with those obtained after injection of Xe-133 into the left ventricular cavity (Bugger 1972) and thus indicate adequate mixing of isotope and blood before the left coronary artery branches. They do not confirm the autoradiographic picture of flow vari-

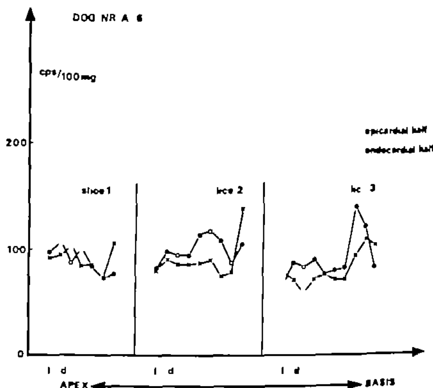


Fig. 1 A representative result of the distribution of Xe-133 in the epi- and endocardial halves of thre slices of the left ventricular wall, after bolus injection of the isotope into the aortic root. The activity of the blocks of each slice is shown the direction from the left anterior descending towards the circumflex branch of the left coronary artery

supplied area The slices are numbered in the direction from the apical region towards the base of the heart The level of activity is constant from slice to slice, and constant in the tissue blocks of each slice independent of blood supply from the anterior descending or circumflex branch of the left coronary artery Fig. 2 shows a comparison between the mean epi- and endocardial activity concentrations (per mCi injected) of all seven dogs exposed to aortic root injection. Each point represents a mean value of about 25 tissue blocks from one heart The slope of the regression line ($b_{y,x}$) is 0.96 ± 0.05 (S D) (which is not different from unity $p > 0.40$) the correlation coefficient 0.99 and the intercept (y 0) 1.2 ± 9.5 (S.D.). The mean ratio of epi- to endocardial activity concentrations was 0.98 ± 0.05 (S E.). No correlation was found by comparing the ratio of epi- to endocardial activity concentrations of each experiment with the time taken to excise the heart

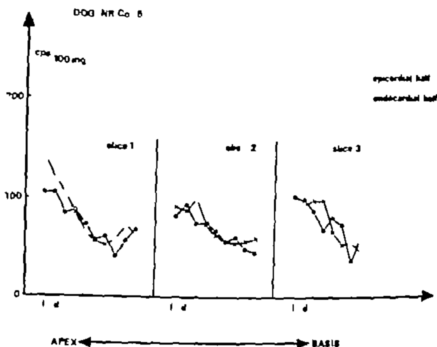
Fig. 3 and Fig. 4 show results obtained after injection of Xe-133 into the left coronary artery in two dogs. Fig. 3 shows an almost equal distribution of activity concentration in the regions supplied by the left anterior descending and circumflex branches, while in Fig 4 there is considerably less activity in the areas supplied by the circumflex branch than in those supplied by the left anterior descending branch Fig. 5 shows a comparison between mean epi- and endocardial activity concentrations (per 0.1 mCi injected) of all seven dogs exposed to left coronary artery injection Each point represents a mean value of about 25 tissue blocks from one heart. The slope of the regression line (b_{y}) is 0.95 ± 0.08 (S.D.)

ently observed by Utley *et al.* (1974) after bolus injection of Xe-133 into the left coronary artery. The level of activity varied considerably from one dog to the other (Fig. 2 and Fig. 5). This was probably due to different fractions of the injected activity reaching the left coronary artery and might be explained by different positions of the tip of the catheter used for the injection.

Injections of Xe-133 into the left ventricular cavity (Bagger 1972) resulted in a 15% higher activity in the endo- than in the epicardial part of the wall. The difference was explained by uptake of xenon through the endocardial surface. This explanation is supported by the uniform distribution achieved by the present aortic root injections.

The activity concentrations measured after injection of xenon into the left coronary artery were not different in apical and basal regions supplied by the same main branch of this artery. In some dogs, however considerably higher activity was found in the area supplied by the left anterior descending branch than in the area of circumflex supply. This non uniform distribution indicates an insufficient mixing of xenon and blood before branching of the left coronary artery probably because of the short or even absent main stem of this artery in dogs (Blair 1961). The uneven distribution between areas supplied by the main branches did not influence the ratio of epi- to endocardial activity concentrations, which was identical to that obtained after aortic root injections. These results indicate that the position of the injection catheter is not crucial if injections of tracers are used for estimation of epi- and endocardial flow distribution in dogs. However its position is critical if dogs are used for regional measurements of flow with scintillation camera, multi-detector systems or autoradiography.

After bolus injection of xenon into the aortic root, the activity transported to each region of the left ventricular wall will be proportionate to flow to that region (Sapirstein 1956). Assuming complete transfer of the isotope from the vascular system to the tissue, a difference in flow between two regions will result in a proportionate difference in initial tissue activities. After injection, during excision of the heart, flow is able to change the activity by wash-out. The amount of activity removed per unit time depends on the volume of distribution of the isotope and the blood flow. The smaller the volume of distribution and/or larger the flow, the faster is the change in tissue activity. In one normal, anesthetized dog the ratio of epi- to endocardial volumes of distribution for xenon was calculated as the ratio of activity concentrations in these regions measured after continuous infusion into the left coronary artery for 8 min. Tauchert *et al.* (1972) have shown that myocardial saturation with argon administered by inhalation is obtained in 4–5 min in normal dogs, while Klassen (1972) found saturation time for tritiated water of about 3 min in normal man. Thus, it is probable that myocardial saturation with xenon was attained in the present expt. and that the slight increase in activity recorded externally during the final part of the infusion was due to uptake in extramyocardial tissue, which could be "seen" by the detector. The uniform epi- and endocardial saturation is in accordance with the general assumption of identical volumes of distribution per unit weight (*i.e.* partition coefficients). The possibility of diffusion of Xe 133 in and from the tissue before and after freezing of the heart has previously been estimated (Bagger 1972). No change in distribution or amount of activity was observed during 15 min before and about 120 min after freezing. Therefore, the initial ratio of



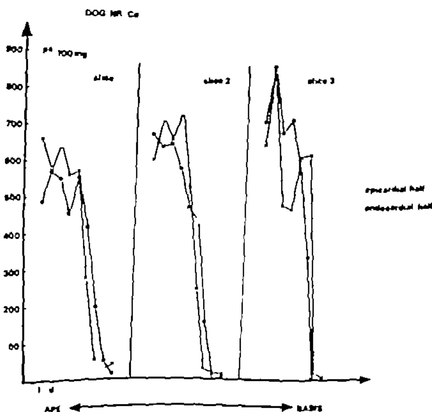


Fig. 3 and 4. The distribution of *Xe-133* in the epi- and endocardial halves of three slices of the left ventricular wall in two dogs, after bolus injection into the left coronary artery plotted in the same way as in Fig. 1

...marily supplies the epicardial half of the left ventricular wall as a consequence of ...dic intramyocardial pressure gradient (Gregg and Eckstein 1941 Brandi and McGre... Baird *et al.* 1970, Van Der Meer *et al.* 1970, Armour and Randall 1971). There... ...form epi- and endocardial mean flows indicate that diastolic flow is larger and ...ic vascular resistance smaller in the endo- than the epicardial region. This might be ...ed by a different caliber of the resistance vessels supplying the two regions (Provenza ...cherle 1959) and/or a difference in their numbers. Cutarelli and Levy (1963) found a ...n distribution of Rb-86 in fibrillating dog hearts perfused at a pressure of 100 mmHg ...with an intraventricular pressure of 5 mmHg. This result does not support the idea of ...cally different vessels supplying the two regions. Thus the difference in vascular ...nce during diastole might be explained by a higher degree of vasodilatation in the ...than epicardial region as suggested by Moir (1972).

Appendix

...ing that the blood flow per unit weight to the epi- and endocardial regions of the left ventricular (f' and f'' respectively) results in initial tissue activities of Xe-133 (A_0' and A_0'') proportionate to flow ...ch of the two halves ... homogeneously perfused, that f' and f'' are different and that the activity as ...on of time in each of the two regions (A_t' and A_t'') follow ... an exponential course (Kety 1949) ...t least ...he first 30 s, then

$$A' = A_0' \exp\left[\frac{-f'}{\lambda}\right] - f'k \exp\left[\frac{-f}{\lambda} t\right] \quad \text{and}$$

$$A_0'' = A'' \exp\left[\frac{-f''}{\lambda}\right] - f''k \exp\left[\frac{-f}{\lambda} t\right]$$

...k ... constant and λ ... the partition coefficient between tissue and blood.
...m these two equations ... follows that

$$\ln f = \frac{f}{\lambda} - \frac{f}{\lambda} - \ln\frac{A}{A'} + \ln f$$

...given values of f', t and λ this equation can be written

$$\ldots rf'' + q \qquad (1)$$

...re the constant ... t/λ and the constant q is $-tf''/\lambda - \ln(A_t'/A'') + \ln f''$
...graphical solution of equation 1 f'' can be determined as the abscissa to the point of intersection between ...functions $y = \ln f''$ and $y = \ldots f'' \ldots q$.

...experimental determined mean ratio of epi- to endocardial activity concentrations (A_t'/A_t'') was 0.98 ± ...0 (S.E.). Therefore the probability that the true mean ratio of the population exceeds 1 10, is less than ...%. Assuming that true A_t'/A_t'' 1 10, $\lambda = 0$ 7 f' ... f'' and $f' = 1$ ml g^{-1} min^{-1} ... 0.25 min (the time ...val between injection of Xe-133 and completion of heart excision) f'' will be 0 87 ml g ... min ... and ...f' ... A_0'/A_0'' 1.15. This means that at normal coronary blood flow the initial ratio of activity concen...tions ...ill be less than 5 % higher than the value measured 15 ... later. Assuming $f' < f''$ the other so...tion of equation 1 would be f'' 6 5 ml g ... min ... which is most unlikely from ... hemodynamic point ...view and incompatible ...ith the smallness of the scatter of the observed values of A_t'/A_t'' relative to the ...mber of the ...values

...his work has been supported by The Danish State Research Foundation 512 192/69.

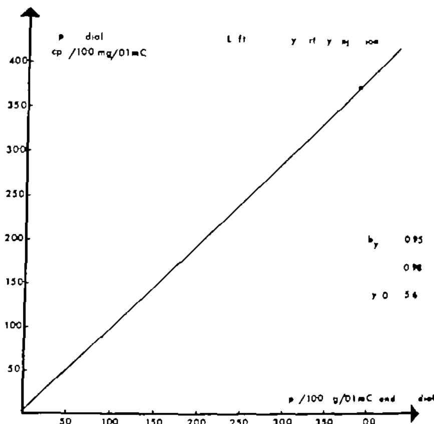


Fig 5 Comparison f mean epi- and endocardial avtivity (cps/100 mg/0 1 mCi injected) of the left ventri lar wall of 7 dogs after intracoronary bolus injection.

epi- to endocardial activity concentrations could only be changed by different epi- a endocardial blood flows in the period used for excision of the heart.

It is shown in the appendix that a normal epicardial blood flow of 1 ml g⁻ min duri a period of 15 s after injection of Xe-133 will result in a reduction of the initial ratio of e to endocardial activity concentrations of less than 5%. This modest reduction due to was out of activity corresponds to the lack of correlation between activity ratios and excisi time in the individual expts Thus, the measured distribution of activity in the left ventric lar wall presumably represents a reliable picture of the flow distribution.

In summary the study has shown that adequate mixing of xenon and left coronary arte blood is obtained by aortic root injections that blood flow is uniform throughout the le ventricular free wall and that intracoronary injections of tracer can result in a false pictu of different flows to the left anterior descending and circumflex supplied areas, due to i adequate mixing, but may be used for estimations of epi- to endocardial flow ratio

The uniform epi- and endocardial distribution of flow is in accordance with results ob tained by infusion of other diffusible tracers (Cutarelli and Levy 1963 Palmer Fam an McGregor 1966, Moir and DeBra 1967 Griggs and Nakamura 1968) and with some of th results obtained with small radioactive microspheres (7–15 μm) (Becker Fortuin and Pit 1971 Buckberg *et al* 1972 and 1975 Becker Ferreira and Thomas 1974 Fixler Saunder and Sugg 1974). Downey and Kirk (1974) have shown that systolic coronary blood flow

dominantly supplies the epicardial half of the left ventricular wall as a consequence of systolic intramyocardial pressure gradient (Gregg and Eckstein 1941 Brandi and McGregor 1969, Baird *et al.* 1970, Van Der Meer *et al.* 1970 Armour and Randall 1971). There, uniform epi- and endocardial mean flows indicate that diastolic flow is larger and diastolic vascular resistance smaller in the endo- than the epicardial region. This might be explained by a different caliber of the resistance vessels supplying the two regions (Provenza and Scherlis 1959) and/or a difference in their numbers. Cutarelli and Levy (1963) found a uniform distribution of Rb-86 in fibrillating dog hearts perfused at a pressure of 100 mmHg and with an intraventricular pressure of 5 mmHg. This result does not support the idea of anatomically different vessels supplying the two regions. Thus the difference in vascular resistance during diastole might be explained by a higher degree of vasodilatation in the endo- than epicardial region as suggested by Moir (1972).

Appendix

Assuming that the blood flow per unit weight to the epi- and endocardial regions of the left ventricular wall (f' and f'' respectively) results in initial tissue activities of Xe-133 (A'_0 and A''_0) proportionate to flow, that each of the two halves is homogeneously perfused, that f' and f'' are different and that the activity as a function of time in each of the two regions (A'_t and A''_t) follows an exponential course (Kety 1949) at least for the first 30 s, then

$$A'_t = A'_0 \exp\left[\frac{-f'}{\lambda}t\right] = f'k \exp\left[\frac{-f'}{\lambda}t\right] \quad \text{and}$$

$$A''_t = A''_0 \exp\left[\frac{-f''}{\lambda}t\right] = f''k \exp\left[\frac{-f''}{\lambda}t\right]$$

where k is a constant and λ is the partition coefficient between tissue and blood.

From these two equations it follows that

$$\ln f'' = \frac{f''}{\lambda}t - \frac{f'}{\lambda}t - \ln\frac{A'_t}{A''_t} + \ln f'$$

For given values of f', t and λ this equation can be written

$$\ln f'' = xf'' + q \quad (1)$$

where the constant $x = t/\lambda$ and the constant q is $-tf'/\lambda - \ln(A'_t/A''_t) + \ln f'$.

By graphical solution of equation 1 f'' can be determined as the abscissa to the point of intersection between the functions $y = \ln f''$ and $y = xf'' + q$.

The experimental determined mean ratio of epi- to endocardial activity concentrations (A'_t/A''_t) was 0.98 ± 0.03 (S.E.). Therefore the probability that the true mean ratio of the population exceeds 1.10, is less than 5 %. Assuming that true A'_t/A''_t = 1.10, λ = 0.7, $f' > f''$ and f' = 1 ml g⁻¹ min⁻¹ as t = 0.25 min (the time interval between injection of Xe-133 and completion of heart excision) f'' will be 0.87 ml g⁻¹ min⁻¹ and f'/f'' = A'_0/A''_0 = 1.15. This means that at normal coronary blood flow the initial ratio of activity concentrations will be less than 5 % higher than the value measured 15 s later. Assuming $f'' > f'$ the other solution of equation 1 would be f'' = 6.5 ml g⁻¹ min⁻¹, which is most unlikely from a hemodynamic point of view and incompatible with the smallness of the scatter of the observed values of A'_t/A''_t relative to the scatter of the f-values.

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References

ANDERSEN H H BAGGER and H GOTZSCHE Non-uniform blood flow in the left ventricular wall of measured by the Xe-133 washout technique. *Acta physiol scand* 1969 76, 376-38..

ARMOUR J A and W C. RANDALL, Canine left ventricular intramyocardial pressures. *Amer J Ph* 1971 220 1833 1839

BAGGER H The distribution of Xe-133 in the left and right ventricular walls of dog hearts after left vent lar bolus injection. *Acta physiol. scand* 1972. 84 75-283

B IRD R J R T MANKTELOW P A SHAH and F M AMELI, Intramyocardial pressure *J thorac ca vasc Surg* 1970. 59 810-823

BECKER L. C. N J FORTUIN and B PITT Effect of ischemia and antianginal drugs on the distributio radioactive microspheres in the canine left ventricle. *Circulat Res*. 1971 28 63-269

BECKER, L. C., R FERREIRA and M THOMAS, Comparison of 86Rb and microsphere estimates of left tricular blood flow distribution *8th Congr Europ Soc Microcirculat* Abstract no 18. France 197

BLAIR E. Anatomy of the ventricular coronary arteries in the dog. *Circulat Re* 1961 9 333-341

BRANDI G W M FAM and M McGREGOR, Measurement of coronary flow in local areas of myocard using xenon 133 *J appl Physiol* 1968 24 446-450.

BRANDI, G and M McGREGOR Intramural pressure in the left ventricle of the dog *Cardiovasc* 1969 3 472-475

BUCKBERG G D D E FIXLER J P ARCHIE and J I E HOFFMAN Experimental subendocardial ische in dogs with normal coronary arteries. *Circulat Res* 1972. 30 67-81

BUCKBERG, G D D E. FIXLER J P ARCHIE R P HENNEY and J I E HOFFMAN, Variable effect heart rate on phasic and regional left ventricular muscle blood flow in the anesthetized dogs. *Ca asc Re* 1975 9 1 11

CUTARELLI R and M N LEVY Intraventricular pressure and the distribution of coronary blood f *Circulat Res* 1963 12 322 327

DOWNEY J M and E. S. KIRK Distribution of the coronary blood flow across the ca ne heart dia systole *Circulat R* 1974 34 251 257

FIXLER, D E K W SAUNDERS and W L. SUGG, Subendocardial underperfusion during acute aort pulmonary shunt g n nesthetized dogs. *Amer Heart J* 1974 87 483-490

FLAMENG W B WÜSTEN B WINKLER B. PASYK W SCHAPER and M CARL, Influence of perfusion p sure and heart rate on local myocard al flow in the collateralized heart with chronic coronary occlus *Amer Heart J* 1975 89 51 59

FORTUIN, N J S K HIARA, L. C BECKER and B. PITT Regional myocardial blood flow the dog stu with radioactive microspheres. *Cardiov sc R* 1971 5 331 336

GREGG, D E. and R W ECKSTEIN, Measurement of intramyocardial pressure *Amer J Ph siol* 1 *132* 781 790.

GRIGGS, D M Jr and Y N KAMURA, Effect of coron ry constriction o myocardial d t bution iodoantipyrine- I *Amer J Physiol* 1968 *215* 1082 1088

H RLEY A J R H RPER nd L H E TES J Distribution of blood flow in the myocard m meas by clearance of enon133 *Circulation* 1966 34 S ppl 3 12.

KETY S. S Measurement of regional circulation by the local clearance of radioactive sod m *Amer H J* 1949 38 321 328

KIRK E S. and C R HONIG Non f rm distribution of blood flow and gradients of o ygen tension the heart *Amer J Phy iol* 1964 207 661 668

KLASSEN, G A Constant infusion of indicators to measure flow nd volume of the left coronary syst of man. In Myocardial blood flow ma Methods nd significance in myocardial d sease (ed A Maseri) *Minerva Medica* Torino 1972 165 172

LOVE W D and G E. BURCH Differences n the rat of Rb86 upt ke by several regions of the myocardi of control dogs and dogs receivi g L-norepinephrine or pitressi *J h Im* 1957 36 479-484

MATHES, P and J RIVAL, Effect of troglycerin on tot l and regional coronary blood flow n the nor and ischaemic myocardium *Cardiovasc R* 1971 5 54-61

MEER, J J VAN DER R. S. RENEMAN, H SCHNEIDER d J WESSELDIJK, A tech ique for est mat on intramyocardial pressure in acute and chronic periment *Cardiov sc Res* 1970. 4 132 140.

MOIR T W Subendocardial distribution f coronary blood flow and the effect of ntianginal dru *Circulat Res* 1972. 30 621-627

[illegible], T W and D. W DeBRA, Measurement of the endocardial distribution of left ventricular coronary blood flow by Rb^{86} chloride. *Amer Heart J* 1965, *69* 795–800.

[illegible], T W and D W DeBRA, Effect of left ventricular hypertension, ischemia and [illegible] drugs on the myocardial distribution of coronary flow *Circulat Res.* 1967 *21* 65–74.

[illegible], W V and C. R. HOWE, Amount and distribution of Rb^{86} transported into myocardium from vascular lumen *Amer J Physiol* 1966, *211* 739–745

[illegible], W H W M FAM and M. McGREGOR, The effect of coronary [illegible] modulatation (Dipyridamole-[illegible]) on the myocardial distribution of tritiated [illegible] *Canad. J Physiol. Pharmacol.* 1966, *44* [illegible]

[illegible], D V and [illegible] SCHREIN, Coronary circulation in the dog heart, demonstration of muscle [illegible] in capillaries *Circulat. Res.* 1959 *7* 318–3.4

[illegible] L. A., Fractionation of the cardiac output of rats with isotopic potassium. *Circulat. Res.* 1956, *4* [illegible]

[illegible], M K KOCHSIEK, H. W HEISS, R. E. STRAUER, D. KETTLER H. D BRETSCHNEIDER, G RAU and H J [illegible], Measurement of coronary blood flow in man by the argon method. In Myocardial blood flow in man. Methods and significance in myocardial disease (ed. by A. Maseri) *Minerva Medica*, Torino 1972 [illegible]

[illegible], J E L. CARLSON, J L E. HOFFMAN, H. M MARTINEZ and G D. BUCKBERG, Total and regional myocardial blood flow measurements with 25 μ, 15 μ, 9 μ and filtered 1 10 μ diameter microspheres and antipyrine in dogs and sheep *Circular Res.* 1974 *34* 391–405.

[illegible], D C, H F HARDMAN, A. R. LADDU, P SOMANI and G. J GROSS, Myocardial distribution of coronary blood flow in the isolated supported heart preparation. *Cardiovasc Res.* 1975 *9* 634–639

[illegible], T W A Donsi Jr P D SCANLON, T J KNOPP and J B. BASSINGTHWAIGHTE, Regional distribution of diffusible tracers and carbonized microspheres in the left ventricle of isolated dog hearts. *Circulat. Res.* 1973, *33*, 573–587

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Evidence for a Rate-Sensitive Regulatory Mechanism in Myogenic Microvascular Control

By

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Abstract

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To reveal a possible rate-sensitive component in the myogenic control, changes of total and segmental vascular resistances in sympathectomized skeletal muscle in response to alteration of vascular transmural pressure (extravascular pressure) by 40 mmHg were compared when the pressure change was applied at two distinctly different rates (15 and 170). The papaverine-dilated vascular bed showed an entirely passive behaviour, whereas the normal, myogenically reactive vascular bed responded with active constriction upon transmural pressure increase and active dilation upon pressure decrease. These responses were especially pronounced in the microvessels where a clearcut two-component effector response was observed. The magnitude of the initial component was distinctly correlated to the rate at which the transmural pressure stimulus was applied, whereas the later steady state component during the static pressure change was rate-independent. At the high rate of pressure increase, the initial rate-dependent microvascular constrictor response was some ten times larger than the steady state response. These observations indicate the existence of a rate-sensitive as well as a static component in the myogenic response to changed transmural pressure, an interpretation strongly supported by a previous analogous study on isolated single-unit vascular smooth muscle (Johansson and Mellander 1975). It is concluded that the microvessels in skeletal muscle are highly responsive to myogenic stimuli and that emphasis should be placed on the dynamic rather than the static characteristics of the stimulus. Such rate-sensitivity in myogenic control would seem to facilitate prompt and proper vascular adjustments, for instance in myogenic autoregulation.

The Bayliss hypothesis (1902) of enhanced contractile activity in vascular smooth muscle in response to increased blood pressure has received strong experimental support in recent years from hemodynamic whole organ and vital microscopy studies (*e.g.* Folkow 1949, 1964, Wiedeman 1963, Mellander *et al.* 1964, Johnson and Wayland 1967, Baez *et al.* 1974). The described active myogenic responses to changes in vascular transmural pressure have been considered of great importance for various autoregulatory phenomena in the local circulatory control, and myogenic mechanisms may also be a major determinant for the development of normal basal vascular tone.

Myogenic reactivity has so far been studied almost exclusively in terms of the active responses which develop upon a given steady state increase or decrease in vascular trans-

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ressure, little attention being paid to the possible effect of the rate at which pressure ged. Such steady state effects might thus be considered to be the result mainly of a myogenic stimulus related to the amplitude of the pressure change. Some recent tudies, however, provided circumstantial evidence for the presence of an additional, ic rate-sensitive component in the myogenic control by the observation of increased vascular tone upon shift from steady to artificial sinusoidal pressure perfusion and Wern 1969) or from steady to normal pulsatile perfusion (Mellander and Arvidsson). Direct evidence for a rate-sensitive component in the vascular myogenic response to changes in length was later obtained from *in vitro* observations of electrical and ical activity in the rat portal vein (Johansson and Mellander 1975) where the muscle tion was exposed to a given static stretch or shortening applied at various rates over the range between −3 and +3% of the muscle length/s. Static stretch by 40 of the length evoked moderate excitatory effects in terms of a 10 to 15 % increase in spike ge above the resting control value and a corresponding increase in active force. ic stretch was much more effective causing graded excitatory responses which at the rates of stretch were up to 20 times more pronounced than those evoked by static . Passive shortening at different rates caused graded inhibitory responses below the level, complete inhibition of electrical and mechanical activity being observed at the negative test value of $\delta L/dt$.

ew of current observations, the myogenically active vascular smooth muscle may be ered to act as mechanoreceptor–mechanoeffector unit in which deformation (e.g. tion), via effects on a pacemaker mechanism, causes facilitation of spike discharge pted to the neighbouring muscle effector cells. The net effect of such mechano-al coupling in response to receptor deformation is changes in the frequency of spike tion and attendant active changes in vascular tone. The mentioned *in vitro* observations ly indicated that myogenic control is dominated by the dynamic rather than the static teristics of the pacemaker stimulus, but before this new dimension in the concept of nic regulation is adopted more universally the principle should be more firmly ented in the *in vivo* situation as well

he present study an attempt was made to test this hypothesis on the sympathectomized ar bed of skeletal muscle by exposing the vessels to increases and decreases in transpressure applied at distinctly different rates. Observations of total vascular resistance segmental resistances in large arterial vessels, microvessels, and large veins were made g the period of phasic change of transmural pressure and also in the steady state phase stant increased or decreased pressure. The results support the concept of an important mic, rate-sensitive component in the myogenic control with special influence on the circulatory resistance vessels *in vivo*. Preliminary reports of this work have been presented elsewhere (Mellander Lundvall and Grände 1976, Mellander 1976, a, b).

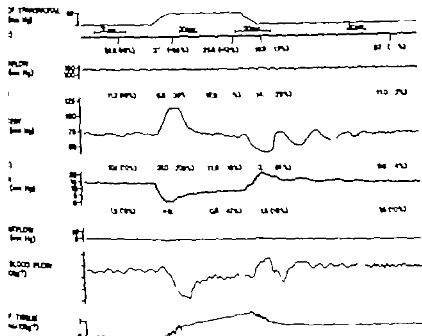
Methods

s of both sexes, weighing 2.8–4.0 kg, were used in the study. The animals were anesthetized with lose (50 mg/kg b) and urethane (100 mg/kg b wt.) after induction with ether. Body temperature maintained at 38 ± 0.5°C throughout the expt.

The investigation was performed on the acutely denervated vascular bed of the right lower leg muscl which were isolated so that the popliteal artery and vein formed the sole vascular connections ith the m part of the body (for details see Lundvall 1972). In brief the skin was dissected free and removed from t lower leg and its muscles were separated completely from the thigh muscles. The paw as removed at t ankle. After heparinization (750 I U /kg b.wt.), blood flow was diverted from the ipsilateral femoral arte to the popliteal artery through a short shunt circuit and the popliteal ein was cannulated and its outfl shunted via tubes to a funnel connected to the right jugular vein. Mean arterial inflow of blood as r corded continuously with an electromagnetic flowmeter (Biotronex) or more frequently ith the use the pressure gradient flowmeter principle. For the latter purpose, a d fferential manometer (Nation Semic. LX1601D) was used for monitoring the pressure drop acros fixed length of the arterial shu The diameter of the shunt was chosen so as to keep the pressure drop small (< 10 mmHg), yet permitti reliable determination f even minute variations of the pressure drop and, hence, blood flow A seri pilot tests of this flowmeter devise demonstrated that it permitted accurate and reproducible flow dete minations over the range encountered in the present expts. Venous outflow of blood as concomitant measured using n optical drop recorder inserted in the venous shunt circuit Mean rterial inflow pressu (below denoted AP) and venous outflow pressure (VP) were measured from T t bes close to the cannulate popliteal artery nd vein. Pressures from small intramuscular arterial vessel ("small artery pressure SAP) and from a small venous vessel ("small vein pressure SVP) were monitored as well. For this purpos the sural artery and vein on the posterior surface of the gastrocnemius muscle were l gated pro inally an flexible nylon tubi gs (d of tip ~0.4 mm) filled with heparinized saline solution were nserted distal t th ligations test expts. showed that an induced pressure change was transmitted with n measurable del through these can ulas. With this technique, the pressure small vessels with n the muscle is transmitte via collaterals to the cannulated vessels, principle for measurement of pressures in the microcirculatio originally described by Haddy *et al* (1954). Frequent checks throughout the expt. ensured free communica tion between the i tramuscular vessels and the cannulas i the sural artery and vein (see Lundvall 1972) Statham pressure transducers were used f the pressure measurements.

After the vascular cannulations were completed, the femur was d vided and plugged and the musc preparation was placed in a f xed position in temperature controlled plethysmograph (37°C), f lled wi Tyrode solution. The entrance of the plethysmograph, through which the arterial and venous cannula passed, was hermetically sealed with flanges and curing silicone rubber (Medical Elastomer Dow Cornin The fluid in the plethysmograph was in communication via a polyethylene t bing ith a reservoir wa placed outside the plethysmograph (f details see Grände *et al* 1974). The hydrostatic fl id pressure insid the plethysmograph, measured at the midlevel of the muscle region with a Statham transducer could b adjusted to desired levels by varying the height of the reservoir n relation to the muscle preparation. Th reservoir was connected to an electronic gravimetric transducer (Grass FT 10) so that fluid displacement between the plethysmograph nd the reservoi caused by cha ges of tissue lume, could be followed continuously In the resting control state, the pressure the plethysmograph was adjusted to the atmos pheric zero level VP in the co trol period was adjusted to a level (4 7 mmHg), gi ing a constant tissu volume (isovolumic state) and was maintained t this level throughout the expt A transcapillary flui equil brium was thus chieved in the co trol period. By lowering the reservoi the pressure in the plethysmo graph and hence tissue pressure, could be decreased I this way all parts of the vascular bed the studied muscle region were exposed to standardized ncrease of vascular transmural pressure (40 mmHg) pplied at two different rates (in 15 and 120 s) Ret rn of the reservoir to the zero level permitted graded decline of vascular transmural pressure at the same standard zed rates. All measured parameters were recorded on a Grass polygraph.

With this experimental approach changes of total vascular resistance a d of segmental resistances in the muscle region evoked by the induced alterat on of vascular tra sm ral pressure co ld be followed (cf Lundvall 1972). From observed alterations of tissue olume the nduced cha ges of regional blood content (capacitance responses) and transcapillary fluid movements could be determined well (see Mellander *et al* 1964 Lundvall *et al*. 1967). Total vascular resistance the muscle region was calculated (AP VP)/ arterial flow The segmental resistances were determined the followi g way Proximal rterial" re sistance = (AP SAP)/arterial flow Micros scular" resistance (SAP SVP) rterial flow (neglect g the minor effect of net transcapillary fluid transfer on blood flow through the xchange nd small enous vessels) And "large ein resistance = (SVP VP) venous outflow Venous outflow (or arterial flow minus recorded capacitance effects and net fluid f ltration) was used for the latter calculation, since tra scapillary fluid movements and transient capacitance effects were induced by changed transmural pressure, implying some difference in the olume flow in arterial and enous essels under these cond tions.



...periment illustrating total and segmental resistance changes in the sympathectomized myogenically ...scular bed of skeletal muscle evoked in response to increased and decreased vascular transmural ...(30 mmHg) applied at high rate (2.7 mmHg/s). Note the pronounced microvascular (R_{micro}) ...r and dilator responses during the periods of phasic increase and decrease in transmural pressure ...uch more moderate constrictor response during the period of constant, increased transmural ... These data indicate an important rate-sensitive component and less pronounced "static" ...t in myogenic control. The arrows below the time scale depict the time at which resistance was ...l. (Paper speed ... during expt. as indicated in time scale.)

Results

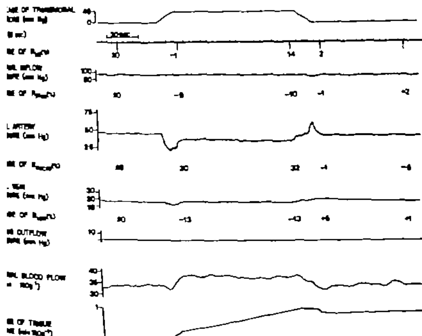
...mpt was made in the present study to investigate whether the resistance vessels ...are myogenically responsive not only to the magnitude of a vascular transmural ...increase or decrease ("static" steady state response) but also to the rate of the ...change ("dynamic" response). For this purpose changes of skeletal muscle vascular ...ce in response to alteration of vascular transmural pressure (extravascular pressure) ...mmHg were compared when the pressure change was applied at two distinctly different ...e., in 15 and 120 s; observations were made under conditions of negligible adrenergic ...or on the normal, myogenically reactive, as well as on the passive, myogenically ..., vascular bed.

...1 shows original recordings from an expt. in which the "normal" vascular bed was ...d to a rapid change in transmural pressure (about 2.7 mmHg/s). Calculated values ...al vascular resistance (R_{tot}), and for segmental resistances, *i.e.* "proximal arterial ...nce" (R_{prox}), microvascular resistance (R_{micro}), and "venous resistance" (R_{ven}) ...ented in the figure (for definitions see Methods and Discussion). In the control period, ...ar transmural pressure was adjusted to the normal level which established a trans-

The investigation was performed on the acutely denervated vascular bed of the right lower leg muscl which were isolated so that the popliteal artery and vein formed the sole vascular connections with the ma part of the body (for details see Lundvall 197?). In brief the skin was dissected free and removed from t lower leg and its muscles were separated completely from the thigh muscles. The paw was removed at t ankle. After heparinization (750 I U /kg b.wt.), blood flow was diverted from the ipsilateral femoral arte to the popliteal artery through a short shunt circuit and the popliteal vein was cannulated and its outfle shunted via tubes to funnel connected to the right jugular ein. Mean arterial inflow of blood as r corded continuously with an electromagnetic flowmeter (Biotronex) or more frequently ith the use the pressure gradient flowmeter principle. For the latter purpose, a differential manometer (Natio Semic. LX1601D) was used for monitoring the pressure drop across a fixed length of the arterial shur The diameter of the shunt was chosen so as to keep the pressure drop small (< 10 mmHg), yet permitti reliable determination of even mi ute variations of the pressure drop and hence, blood flow A series pilot tests of this flowmeter devise demonstrated that it permitted ccurate and reproducible flow dete minations over the range encountered in the present expts. Venous outflow of blood was concomitant measured usi g an optical drop recorder inserted n the ven us shunt circuit. Mean arterial inflow pressu (below denoted AP) and venous outflow pressure (VP) were measured from T-tubes close to the cannulate popliteal artery and vein. Pressures from a small intramuscular arterial vessel ("small artery pressure SAP) and from small venous vessel ("small vein pressure SVP) were monitored as well. For this purpose the sural artery and vein on th posterior surface of the gastrocnemius muscle were ligated proximally an flexible nylon tubings (i d of tip ~ 0.4 mm) filled with heparinized saline solution were inserted distal to th ligations test expts. showed that n induced pressure change was transmitted w th no measurable dela through these cannulas. With this technique, the pressure in small vessels with n the muscle is transmitted via collaterals to the cann lated vessels, a principle f measurement of pressures n the microcirculation riginally described by Haddy *et al* (1954) Frequent checks throughout the expt. ensured free communica tio between the intramuscular vessels and the cannulas the sural artery and ei (see Lund all 1972) Statham pressure transducers were used f the pressure measurements.

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...imilar expt. to that in Fig. 1 performed on the papaverine-dilated muscle vascular bed. Note the ... passive behaviour of all resistance sections in response to changed transmural pressure in this expt. ... myogenic reactivity was abolished.

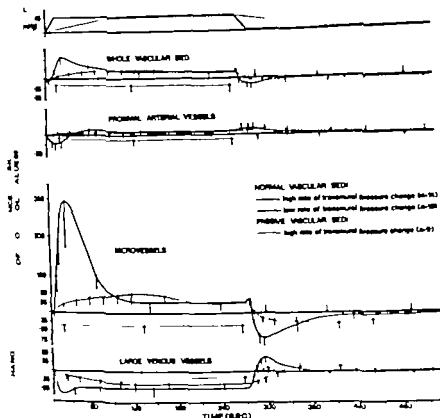
... for the venous one. For reasons discussed below this active constrictor response in all ...ood is essentially myogenic in nature. This constrictor mechanism, however was much ...effective during the phase of the transmural pressure change than in the later phase of ...nt, increased pressure, which might indicate the presence of an initial rate-sensitive ...later static component in the myogenic vascular control. If this interpretation is correct, ...would expect the rate-sensitive component of the myogenic response to be less pro-...ced if the myogenically reactive vascular bed was exposed to the same transmural ...re change, *i.e.* 40 mmHg, but applied at a significantly lower rate than in Fig. 1 ... 3 shows original recordings from the same preparation as in Fig. 1 but the ascular ...as here exposed to low rate transmural pressure change (0.33 mmHg/s). It can be ...that the pattern of vascular response in essential parts was qualitatively similar to that ...ed by the rapid transmural pressure change in Fig. 1 but that the responses during the ...ure change, as predicted above, were quantitatively much less pronounced. Thus, t...ime of maximum response, *i.e.* at the completion of the pressure rise, R_{tot} was now ...ased by only 23% and R_{micro} by only 55% above the values in the control period com-...d to 65% and 208% in Fig. 1. R_{prec} in Fig. 3 was virtually unchanged at this time, ...as the venous compartment showed roughly the same degree of passive dilation as in ... 1. The vascular responses in the steady state phase of elevated transmural pressure, ...e other hand, were practically identical in Figs. 1 and 3. When transmural pressure was ...ased at the low rate of 0.33 mmHg/s (Fig. 3), the vascular effects were similarly much

capillary Starling fluid equilibrium as indicated by the isovolumic tissue volume cur
Control pressures and blood flow were constant except for minor normal cyclic variatio
Arterial inflow pressure and venous outflow pressure were not significantly affected by t
subsequent changes of transmural pressure but other marked vascular reactions were i
duced. Thus, during the 15 s period of transmural pressure rise, blood flow first showed
transient slight increase, but within few seconds it decreased rapidly to attain a minimu
value very shortly after the completion of the pressure rise. These observations demonstra
a transient passive dilation followed quickly by a constrictor response of the resistan
vessels, which increased total vascular resistance to a level about 65 above that in tl
control period. The segmental resistances also changed markedly as evidenced by the co
comitant changes of flow SAP and SVP Thus, few seconds after the beginning of tl
transmural pressure rise, SAP increased and SVP decreased rapidly maximal changes bei
reached just after completion of the transmural pressure rise. At this time microvascul
resistance had increased by about 210 % above the control level, whereas proximal arteri
resistance had decreased by almost 40 % and there was also a fall of venous resistanc
initially to quite a low level Some 10 s after the level of constant, high transmural pressu
was reached, blood flow and SVP again rose and SAP decreased gradually to attain ne
steady state values within about two minutes. At this time, R_{tot} and R_{micro} were only mo
erately increased (by 12 and 18% above control), whereas R_{ven} stabilized at a level son
50 below the control value. R_{prox} had now increased slightly above the control level (t
15).

During the subsequent decrease of transmural pressure back to the control level at a ra
of −2.7 mmHg/s (Fig. 1) the pattern of vascular response was opposite to the one durir
the pressure rise, insofar that blood flow and SVP increased and SAP decreased rapidl
At completion of the pressure fall R_{tot} and R_{micro} were significantly lower than in the init
control period, *i.e.* by 13 % and 61 respectively indicating clearcut dilator effects. R_{pr}
at the same time had further increased somewhat whereas R_{ven} approached the contr
value. Some 10 s after the return to the control transmural pressure level, blood flow an
SVP again decreased and SAP increased markedly after which followed an oscillato
behaviour gradually damped out in 2–3 min when control values for blood flow R_{tot}
R_{prox}, R_{micro}, and R_{ven} were reached

Fig. 2 shows the effects in the maximally dilated vascular bed of the same transmur
pressure alterations as in Fig. 1 Vascular tone and myogenic reactivity in this preparatio
was abolished throughout the observation period by a close arterial infusion of a large do
(100 mg/kg tissue) of papaverine. It can be seen that this vascular bed showed a passiv
behaviour in all resistance sections as indicated by the calculated figures for the per ce
decrease in total, proximal arterial, microvascular and venous resistance below contr
during the transmural pressure increase, and the quite rapid return to control resistanc
values upon the subsequent pressure decline.

An increase of transmural pressure in the maximally dilated vascular bed (Fig.) thu
caused the expected effects of passive vessel distension in all resistance sections, whereas th
vascular bed with normal tone (Fig. 1) showed clear signs of some mechanism which effe
tively counteracted such distension and even caused pronounced constriction in all section



[Fig.] 4 Compiled data for total and segmental resistance changes in the normal, myogenically reactive and the [passive], myogenically inactive vascular bed of skeletal muscle evoked in response to increased and [reversed] vascular transmural pressure (40 mmHg) applied at high (2.7 mmHg/s) and low (0.33 mmHg/s) [rates. For] the symbols see key [illegible] number of observations) *Note the presence of both* [illegible] *rate-dependent* and [rate]-independent (steady state) resistance responses in the microvessels of the normal vascular bed and the [passive] behaviour of the myogenically inactive [v]ascular bed. (The period of constant high transmural [pressure] lasted in all experiments about 2.5 min, implying some extrapolation in time of the steady state [illegible] depicted by the solid line curves.)

[Resis]tance in all vascular sections during the whole period of increased transmural pressure. [In] the [v]ascular bed with preserved myogenic tone (solid and dashed curves), a passive disten[sion] effect of the transmural pressure rise was maintained only in the venous section but was [v]ery transient in the others. Thus, within few seconds, resistance exceeded the control value [in] the overall resistance [v]essels and the microvessels and, with some longer delay this also [occ]urred in the proximal arterial vessels, indicating active constrictor responses in these [s]ections during raised transmural pressure. In the overall resistance vessels and in the micro[v]ascular section, the constrictor responses reached clearcut peak values just after completion of the transmural pressure rise. However the magnitudes of these constrictor responses were much greater with the high (2.7 mmHg/s) than the low (0.33 mmHg/s) rate of the transmural pressure increase. The later steady state constrictions (reached about 2 min after

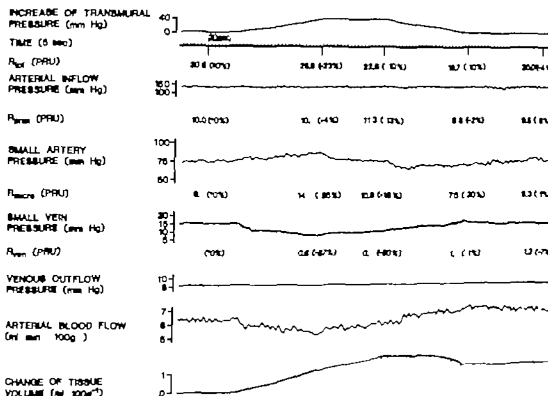


Fig. 3 Experiment illustrating total and segmental resistance changes the sympathectomized myogenically reactive vascular bed f keletal muscle evoked n response to increased and decreased ascular transmural pressure (40 mmHg) applied t *low* rat (0 33 mmHg/s). Note the d minution of the "rate-sensiti e but not of the static component in the microvascular myogenic resistance response compared th the resistance effect t high rate of pressure change shown n Fig. 1

less pronounced than during the rapid pressure decline (Fig. 1) recovery to control vascular tone *after the pressure decline was achieved in both cases* within 2-3 min, but in Fig. 3 without the pronounced oscillatory behaviour exhibited after the rapid transmural pressure decline.

Fig. 4 summarizes the results from similar expts. performed on 11 skeletal muscle preparations which, after completed surgery from a technical point of view could be considered satisfactory The data are given *in terms of total and segmental vascular resistance changes* expressed in per cent of the control values prevailing before the transmural pressure increase (*cf* Fig. 1-3) The key in Fig. 4 defines the three different types of experiments and also the number of serial observations (n) The data in the diagrams refer to mean values ±S D and the curves connecting the different points are fitted by eye and smoothed out neglecting minor resistance variations *between the different points. Table I shows the mean* PRU values ±S D for total, proximal arterial microvascular and venous resistances in the initial control period (corresponding to time zero in Fig. 4) for the three different types of expts. It can be seen from Fig. 4 that, for the whole material, the three patterns of resistance response to changed transmural pressure much resembled those already described (Fig. 1-3) The following main conclusions *may be drawn The vascular bed with abolished* tone (dotted curve) showed an entirely passive behaviour (distension) in terms of decreased

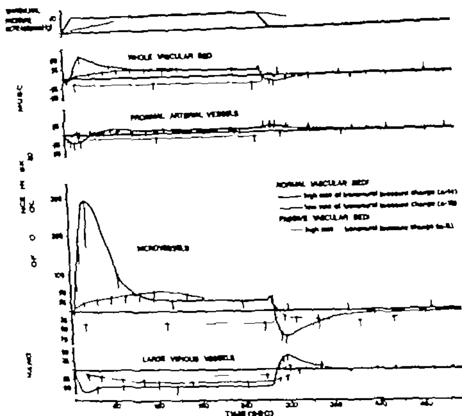


Fig. 4 Compiled data for total and segmental resistance changes in the normal, *myogenically* reactive and the passive, myogenically inactive vascular bed of skeletal muscle evoked in response to increased and decreased vascular transmural pressure (40 mmHg) applied at high (2.7 mmHg/s) and low (0.33 mmHg/s) rates (for symbols see key; number of observations). Note the presence of both rate-dependent and rate-independent (steady state) resistance responses in the microvessels of the normal vascular bed and the passive behaviour of the myogenically inactive vascular bed. (The period of constant high transmural pressure lasted in all experiments about 2.5 min, implying some extrapolation in terms of the steady state data depicted by the solid line curves.)

resistance in all vascular sections during the whole period of increased transmural pressure. In the vascular bed with preserved myogenic tone (solid and dashed curves), a passive distension effect of the transmural pressure rise was maintained only in the venous section but was very transient in the others. Thus, within few seconds, resistance exceeded the control value in the overall resistance vessels and the microvessels and, with some longer delay, this also occurred in the proximal arterial vessels, indicating active constrictor responses in these sections during raised transmural pressure. In the overall resistance vessels and in the microvascular section, the constrictor responses reached clearcut peak values just after completion of the transmural pressure rise. However, the magnitudes of these constrictor responses were much greater with the high (2.7 mmHg/s) than the low (0.33 mmHg/s) rate of the transmural pressure increase. The later steady state constrictions (reached about 2 min after

TABLE I Mean control values ±S.D for total and segmental vascular resistances (PRU units ml^{-1} 100 g tissue^{-1}) in skeletal muscle in the three types of experiments illustrated in Fig. 4.

Control PRU	Normal vasc. bed High rate pressure change	Normal vasc. bed Low rate pressure change	Passive vasc. bed High rate pressure change
R_{tot}	19.0±6.4	17.6±6.1	4.3±1.4
R_{prox}	11.6±3.9	10.3±3.1	3.1±1.4
R_{micro}	6.4±3.7	6.4±2.8	0.6±0.4
R_{ven}	1.0±0.7	0.9±0.8	0.6±0.4

the peak) on the other hand, were about equal at both rates. In the proximal arterial vessel the constriction was slower in onset and it raised resistance in the steady state to a level jus barely above the control value.

When *transmural pressure was decreased in the vascular bed with abolished tone*, a vascular sections showed a passive behaviour the resistances quickly returning to contro values after a transient overshoot In experiments with preserved vascular tone, such behaviour was noted in the venous and to some extent also in the proximal arterial section whereas the overall and microvascular resistance vessels exhibited clearcut active dilato responses in terms of significantly decreased resistance compared to the preceding and al to the control values. Note that these dilator responses, reaching their maxima soon afte the completed pressure decline, were much more pronounced with high than low rate o pressure decrease. Few minutes later all resistances had returned to their initial contro levels.

The same type of expt. as depicted by the solid line in Fig. 4 was performed on 3 *of th cats before and after complete α-adrenoceptor blockade with phenoxybenzamine (15 mg* kg^{-} muscle tissue) The effectiveness of the blockade was tested with a large dose (10 μg kg^{-} *muscle tissue) of noradrenaline given close arterially* The vascular constrictor effect of increased transmural pressure were practically identical before and after the blockade indicating that they were not mediated by an α-adrenergic mechanism.

The results described so far seem to strongly indicate the presence of a rate-sensitive ("dynamic") component in the myogenic vascular control causing excitatory (constrictor) responses, especially in the microvessels, during the phase of increasing transmural pressure and inhibitory *(dilator) responses during the phase of the pressure decline*. The magnitude of the dynamic response was graded in relation to the rate of the pressure change further its onset occurred within few seconds, and it seemed to be fully developed within 15–20 s irrespective of the rate of the pressure change (see *Fig. 1 3 and 4). The data also support* the view of a "static" component in the myogenic control apparently related to the magnitude of the transmural pressure increase (see below) and revealed as a moderate steady state constrictor response in the microvessels and the proximal arterial vessels during the phase of constant, increased pressure. The results might also warrant the conclusion that the microvessels are *more* sensitive to "dynamic" than "static" myogenic stimuli, whereas the proximal arterial vessels may be dominated in their control by the latter type. Large venous vessels, on the other hand, seem to be little, if at all, controlled by myo[illegible]anisms.

It should be emphasized that our whole material consisted of 14 cats, but, after completed surgery 3 preparations were considered unsatisfactory because of technical failure, such as hypothermia or major accidental bleedings, leading, among other things, to arterial hypotension well below the normal level. It is well known from previous studies (e.g. Folkow 1949 Lundvall et al. 1967) that such preparations are largely devoid of myogenic reactivity a fact which was confirmed on these three preparations. It was therefore considered justified to exclude results from these experiments in the material presented in Fig. 4, but still they should be reported. These preparations, in contrast to the others, characteristically showed quite marked dilation in all resistance sections during the phase of the transmural pressure increase, but there was a slight tendency of gradual return of resistance towards the control level in the proximal arterial vessels and in the microvessels in the following steady state phase of constant, increased pressure. It thus appears that these preparations were largely insensitive to myogenic stimuli, at least to "dynamic" ones. Whatever the explanation of the failing reactivity these experiments can help to exclude the possibility that rheological effects of transcapillary plasma fluid loss by viscosity changes or by sludging could have contributed to the vascular resistance changes in the reactive vascular bed (Fig. 4). Since the typical resistance increase depicted in this figure was lacking in these three preparations despite the fact that fluid filtration (0.75 ml min⁻ 100 g⁻¹) as well as blood flow were in the same order of magnitude in all experiments, rheological factors could hardly have been responsible for the results presented in Fig. 4.

Discussion

Local intrinsic vascular regulation is considered to be accomplished mainly by the myogenic and the chemical metabolic control systems (e.g. Mellander and Johansson 1968, Johnson 1974). There is much evidence to indicate that both these mechanisms by interaction are importantly involved in autoregulatory phenomena, such as autoregulation of blood flow (e.g. Folkow 1962, Berne 1964, Johnson 1964) and of capillary hydrostatic pressure (e.g. Björnberg and Mellander 1974).

For a specific study of myogenic reactions to changed transmural pressure, any significant alteration of blood flow must be avoided, since this leads to secondary interference with the metabolic control system. Observations of myogenic responses to changes in arterial inflow pressure or venous outflow pressure are therefore quite difficult to interpret because of the attendant variation of flow. With the present plethysmographic approach by which the whole vascular bed could be exposed to standardized change of vascular transmural pressure by alteration of extravascular pressure, such problems were largely overcome. Strong and precisely graded transmural pressure increases or decreases could thus be applied without affecting the arterio-venous pressure difference. Hence, blood flow was not primarily altered, except for the effects of passive vessel distension or passive elastic recoil, but such flow effects, as shown by the results (Fig. 1 and 3), were almost negligible. It is therefore believed that influences from the metabolic control system can be largely disregarded in the interpretation of the described constrictor responses evoked by the transmural pressure increase and the dilator responses evoked by the pressure decrease. Since autonomic nervous

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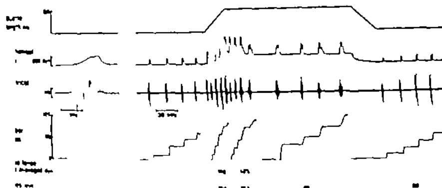
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The same type of expt. as depicted by the solid line in Fig. 4 was performed on 3 of cats before and after complete α-adrenoceptor blockade with phenoxybenzamine (15 m kg⁻ muscle tissue). *The effectiveness of the blockade was tested with a large dose (10 μ* kg⁻ muscle tissue) of noradrenaline given close arterially The vascular constrictor effe of increased transmural pressure were practically identical before and after the block indicating that they were not mediated by an α-adrenergic mechanism.

The results described so far seem to strongly indicate the presence of a rate-sensit (dynamic") component in the myogenic vascular control causing excitatory (constrict responses, especially in the microvessels, during the phase of *increasing transmural pressu* and inhibitory (dilator) responses during the phase of the pressure decline The magnit of the dynamic response was graded in relation to the rate of the pressure change furth its onset occurred within few seconds, and it seemed to be fully developed within 15–20 irrespective of the rate of the pressure change (see Fig. 1 3 and 4) The data also supp the view of a "*static*" component in the myogenic control, apparently related to the mag tude of the transmural pressure increase (see below) and revealed as a moderate steady st constrictor response in the microvessels and the proximal arterial vessels during the pha of constant, increased pressure. The results might also warrant the conclusion that the micr vessels are more sensitive to "dynamic" than static" myogenic stimuli, whereas the proxim arterial vessels may be dominated in their control by the latter type. Large venous vesse on the other hand, seem to be little if at all controlled by myogenic mechanisms.



5 Effects of dynamic and static stretch and shortening on mechanical and extracellularly recorded [illegible] activity in the isolated rat portal vein. Lengthening from 6 0 to 8 5 mm at rate of 5 mm/min [illegible] marked excitatory effects, followed by an afterdischarge (see calculated data for active force and [illegible] time). At constant increased length, activity stabilized at levels only somewhat above control [illegible] initial length. Phasic shortening at the rate of − 5 mm/min caused complete abolition of activity [illegible] control length was reached. Left panel shows high paper speed recordings during single burst of [illegible]. (Reproduced from Johansson and Mellander Circulat Res. 36 76–83 1975, by permission.)

[illegible] stretch and shortening on mechanical and extracellularly recorded electrical activity [illegible] portal vein. Lengthening by 40% from 6 0 to 8.5 mm at a constant rate (dL/dt ~ 5 mm/ [illegible] 13% of the muscle length/s) evoked marked excitatory effects during the period of [illegible] stretch, followed by an afterdischarge (see calculated data for active force and [illegible] time). During the period of static stretch at the constant increased length, activity [illegible] at a level only somewhat above the control value at initial length. Phasic shortening [illegible] rate of 5 mm/min caused complete abolition of electrical and, hence, mechanical [illegible] but control activity reappeared when the muscle had reached its initial control length. [illegible] left panel shows high paper speed recordings of a single spontaneous contraction in the [illegible] period, the corresponding burst of spikes, and the spike-counter output. The study [illegible] showed that the magnitudes of the electrical and mechanical responses to dynamic [illegible] were distinctly graded in relation to the rate of stretch over a wide range, both for [illegible] and negative values of dL/dt (Johansson and Mellander 1975).

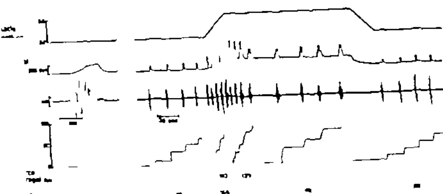
[illegible] findings provided direct support not only for the myogenic hypothesis but also for [illegible] presence of a "rate-sensitive" as well as a "static" component in the myogenic control, [illegible] which the former was shown to be the much more effective one. From a subsequent *in* [illegible] analysis (Sigurdsson *et al* 1977), it appeared that the rate-sensitive myogenic response [illegible] more closely correlated to the change in passive force (dP/dt) than to dL/dt. It was also [illegible] demonstrated that the rate-dependent myogenic response was equally pronounced when [illegible] shortening could occur as under conditions when contraction manifested itself [illegible] as active force ("isotonic" and "isometric" contractions). Both these observations [illegible] be important when considering the *in vivo* situation in view of the facts that passive [illegible] distension effect was not prominent feature in the microvessels during transmural pressure [illegible] (see below) and, further that smooth muscle contraction in resistance vessels as a [illegible] implies active shortening. There is every reason to believe that excitatory and inhibitory

mechanisms did not seem to be involved these vascular reactions most likely can be considered to be myogenic in nature.

However metabolic influences possibly could have attenuated the myogenic responses, once they were developed, as blood flow decreased below the control level during the transmural pressure increase and increased above the control level during the pressure decrease (*e.g* Fig. 1). Such an influence thus might have contributed to the rapid decline of the initial myogenic constrictor response to the transmural pressure increase (Fig. 1 4). These flow effects were relatively moderate, however and the metabolic influence must have been quite small in view of a recent finding of Johnson *et al* (1976) that even *complete* flow cessation by arterial occlusion did not cause any significant metabolic vasodilation in skeletal muscle unless the duration of the occlusion period exceeded 30 s.

The myogenic responses described in this study (*e.g* Fig. 4) occurred mainly in the "microvessels" delineated by the sites along the vascular tree from which small artery pressure and small vein pressure, via collaterals, were monitored. The dimensions of these microvessels on the arterial side can be roughly deduced from data for simultaneous pressure and diameter measurements in the muscle microvessels reported by Fronek and Zweifach (1975) The average value for small artery pressure in the control period of 63 ± 15 (S D) mmHg in our material thus implies that it was monitored from arterial vessels in the order of 15-20 μm It is known from the mentioned study and others that arterial microvessels of this size and smaller exhibit the greatest responsiveness to several stimuli and the present observations indicate that myogenic reactivity is also mainly confined to these vessels. It cannot be excluded however that the minute venous vessels to some extent contributed to the observed myogenic microvascular effects It should be noted that the microvascular responses were strong enough to clearly affect total vascular resistance despite the fact that the large veins behaved passively

Our interpretation of the present results in terms of a "rate-sensitive and a "static component in the microvascular myogenic control is mainly based on the finding of a clear cut two-component effector response, the initial one showing distinct quantitative correlation to the rate at which the transmural pressure stimulus was applied, and being temporally separated from the steady state component evoked during the period of constant increased or decreased pressure the magnitude of the latter component seemed to be pressure amplitude dependent Some alternative explanations were excluded by special test expts. (see Results). The presence of rate-sensitivity in the myogenic control has previously been suggested from results obtained by other *in vivo* approaches mentioned in the Introduction (Basar and Weiss 1969 Mellander and Arvidsson 1974) Strong arguments for our proposed interpretation can be developed from recent knowledge of vascular smooth muscle physiology In this respect an analogous *in vitro* study by Johansson and Mellander (1975), in which electrical and mechanical myogenic responses in the rat portal vein to passive stretch were examined in detail, deserves special attention and will be briefly reviewed. The smooth muscle of the portal vein is of the single-unit propagating type and such behaviour is considered to be a dominant characteristic also of the smooth muscle in precapillary microvessels (Mellander and Johansson 1968, Somlyo and Somlyo 1968 Bevan and Ljung 1974) Fig. 5 is a reproduction of one of the mentioned *in vitro* expts. It shows (right panel) the effects of "dynamic" and "static"



Effects of dynamic and static stretch and shortening on mechanical and extracellularly recorded l activity in the isolated rat portal vein. Lengthening from 6.0 to 8.5 mm at a rate of 5 mm/m marked excitatory effects, followed by an afterdischarge (see calculated data for active force and nt time). At constant increased length, activity stabilized at levels only somewhat bove control l initial length. Phasic shortening at the rate of 5 mm/min caused complete bolition of activity itial length as reached. Left panel shows high paper speed recordings during ingle burst of Reproduced from Johansson and Mellander, *Circulat Res*. 56, 76–83 1975 by permission.)

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contractile responses of vascular smooth muscle *in vitro* have a corollary in the *in vivo* situation in terms of vasoconstriction and vasodilation, respectively

In view of these considerations and of the conspicuous similarity between the pattern of resistance response illustrated in Fig. 4 and the pattern of vascular smooth muscle contractile response depicted in Fig. 5 it seems most likely that the results of the present study can be interpreted in terms of a "rate-sensitive" as well as a "static" component in the myogenic microvascular control *in vivo*

It follows that the magnitude of the rate-dependent vascular constrictor response to the transmural pressure increase in the present material (Fig. 4) can be roughly estimated by subtracting the steady state resistance response from the initial peak resistance response. Such deductions suggest that the rate-dependent microvascular constrictor response was roughly eight times greater at the high (2.7 mmHg/s) than the low (0 33 mmHg/s) rate of the transmural pressure increase (*cf* solid and dashed curves in Fig. 4). The corresponding difference with regard to the overall resistance vessels in the region was in the order of four to fivefold At the high rate (solid curve in Fig. 4), the rate-dependent "dynamic" microvascular response was more than ten times greater than the "static" response. Note also the promptness of the "dynamic" responses at both rates, being triggered almost immediately upon the transmural pressure change and fully developed within some 20 s (Fig. 1 3 and 4)

As mentioned in the Introduction, myogenic activity in vascular smooth muscle is considered to be controlled by a "pacemaker" mechanism which in response to some stimulus related to raised transmural pressure, causes increased impulse discharge propagated to the neighbouring muscle effector cells and, hence, vasoconstriction. The *in vitro* expts. suggested that the sensor element is some kind of a mechano-receptor (Johansson and Mellander 1975 Sigurdsson *et al* 1976, 1977) responsive to "static" as well as "dynamic" stimuli (ΔL and dL/dt, or ΔP and dP/dt respectively)

The situation *in vivo* is complicated for instance by the fact that the myogenic constrictor response to a given stimulus results in decrease of vessel calibre below the control size (as confirmed by the present results, Fig. 4) which *a priori* would seem to abolish the stimulus, at least if the receptor is responding to increased length. In fact, Fig. 4 indicates that the initial passive distension of the microvessels upon the transmural pressure increase (as judged from the observed decrease in resistance) was so small and shortlasting that it possibly could not have been responsible for the much more prolonged "dynamic" and "static" constrictor responses. If as suggested by Johnson and Wayland (1967) and Johnson (1974), the sensor element is coupled in series with the contractile elements, receptor elongation might still be maintained during myogenic vessel constriction An alternative explanation was proposed by Folkow (1964) implying static elongation beyond control length of the sensor element during the period of relaxation of the smooth muscle between repetitive rhythmic contractions. It appears that the present concept of a rate-sensitive sensor can aid in the understanding of maintained receptor stimulation during constriction If the "static" myogenic constrictor response evoked during the plateau of a pressure increase fails to maintain vascular tone at the new increased level the vessels would again be transiently distended which, in turn, would create the stimulus for a repeated "dynamic" constrictor response. Note in this context that the "dynamic" stimulus (*e.g* dL/dt) in contrast to the

"static" one, is largely independent of absolute sensor length. It is quite possible then that vascular tone during increased transmural pressure will oscillate around an increased level as a result mainly of repetitive dynamic sensor stimulation. Similar events might occur during pressure decrease (possibly explaining the oscillations in Fig. 1). The damping (perhaps especially pronounced at high pressure) and the asynchrony of such cyclic variations in tone are, however, factors which could contribute to the maintenance of an overall relatively stable level of blood flow in the tissue, as usually observed in the steady state in the present expts.

It might be argued in this context that the rapid decline of the initial constrictor response to transmural pressure elevation (Fig. 4) is just a reflection of an eliminated "static" receptor stimulus upon vessel constriction and that no "dynamic" stimulus need be invoked. This possibility seems to be refuted by several of the arguments given above, for instance the findings that the initial response was distinctly correlated to the rate of the transmural pressure increase and that the dynamic response in vitro during active muscle shortening was equal to that during isometric force development (Sigurdsson *et al.* 1976, 1977).

The functional significance of the suggested rate-sensitive component in the myogenic control might be manifold. In general it would seem that, like any servo-mechanism, the myogenic control system would function more properly with receptors sensitive not only to static deformation but also to the rate of deformation. In a linear system, such a servo-mechanism would be much refined by the thereby increased order of the transfer function. With appropriate characteristics of the transfer function (cf. Melsa and Schultz 1969) the system would show damped oscillatory behaviour. Perhaps this could be an additional explanation of the oscillations, especially in the microvessels, noted upon the transmural pressure decline in Fig. 1. For rapid servocontrol, as implicit in myogenic flow autoregulation, a rate-sensitive receptor mechanism might well be required. It would imply that the quicker the transmural pressure increases, the more effective and prompt is the active constriction that counteracts the expected passive vessel distension. Energy expenditure would be much greater if effective constriction were delayed more or less to the moment full passive distension already has developed, especially in view of the effect of Laplace's law which imposes an extra load due to the then much increased passive wall tension.

The model of study used here with an equal increase of transmural pressure across the whole vascular bed resembles the situation in dependent regions when one shifts from supine to erect body posture. The initial load on the dependent vascular bed caused by kinetic energy exceeds several times the later steady state hydrostatic load and the overshoot in microvascular resistance above control noted in the present expts. (Fig. 4) might then be needed to protect the vessels against damage and to cause subsequent proper flow autoregulation. The concept of a dynamic component in the myogenic control can also explain how prompt myogenic vasoconstrictions and vasodilatations are accomplished during the phase period of an arterial blood pressure change to compensate quickly for the altered arterio-venous pressure difference and how such responses are graded in relation to the rate of the pressure change.

It has sometimes been said that a positive feedback mechanism is inherent in the myogenic theory (the pressure rise evokes constriction and an increase in resistance which, in turn,

causes an additional pressure rise, etc.) The *in vitro* studies (Johansson and Mellander 197 Sigurdsson *et al* 1976) strongly indicated that any extreme effects of such a mechanism w not ensue, since there was an upper biological limit of the dynamic response at high rates stretch. Furthermore, the chemical metabolic control system acts as a brake on the myogen control system, since intense vasoconstriction is counteracted by the attendant local acc mulation of vasodilator metabolites (Berne 1964) The dynamic component in myogen control might also as mentioned, favour the development of a damped oscillatory respon of the vascular smooth muscle when transmural pressure is changed with consequent a ternating periods of excitation and inhibition caused by alternation between repetitive pos tive and negative rate-dependent stimuli. This implies an inherent negative feedback com ponent in the myogenic control which efficiently might hinder extreme myogenic effecto reactions.

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References

BAEZ, S., Z. LAIDLAW and L. R. ORKIN, Localization and measurement of microvascular and microci culatory responses to venous pressure elevation in the rat. *Blood Vessels 1974 11* 60– 76.

BASAR, E. and C. WEISS, Rate sensitivity of the mechanism of pressure induced change of vascular resistance *Kybernetik* 1969 5 41 247

BAYLISS, W M., On the local react ons of the rterial wall to changes in internal pressure J Ph sio (Lond.) 1902. 28 220–231

BERNE R. M Regulatio of coronary blood flow *Physiol. Rev* 1964 44 1 79

BEVAN J A and B. LJUNG, Longitudinal propagation of myogenic activity in rabbit rteries and n the ra portal vein. *Acta physiol. scand* 1974 90 703–715

FOLKOW B., I travascular pressure as a factor regulating the tone of the small vessels. *Acta physiol scand* 1949 17 89–310.

FOLKOW B Transmural pressure nd ascula tone—some aspects of old controversy *Arch int Pharmacodyn*. 1962. 139 455–469

FOLKOW B., Description of the myogenic hypothesis. *Circulat Res*. 1964 15 Suppl 1 79–285

FRONEK, K and B. W ZWEIFACH, Microvascular pressure distribution skeletal muscle nd the effect of vasodilation. *Amer J Physiol* 1975 2 8 791 796.

GRÄNDE, P-O J JÄRHULT nd S. M LLANDER, Method for gravimetric registration of cha ges a t sue volume *Act physiol scand*. 1974 91 211 215

H DDY F J A. G RICHARDS, J L ALDEN and M B VISSCH R Small vei nd rtery pressures in normal and edemat us extremities of dogs under local and general anaesthesia. *Amer J Physiol* 1954 176 355–360.

JÄRHULT J nd S. MELLANDER Autoregulation of capillary hydrostatic pressure skeletal muscle during regional arterial hypo- and hypertension *Acta physiol sc nd* 1974 91 32–41

JOHANSSON, B. and S MELLANDER, Static and dynamic component t the ascular myogenic response to passive changes length as revealed by electrical and mechanical record ngs from the rat portal ve. *Circulat Res*. 1975 36. 76–83

JOHNSON, P C. (ed.) Symposium on utoregulat o of blood flow *Circulat Res* 1964 15 Suppl 1 1 291

JOHNSON, P C., The microcirculation, and local nd humoral control of the circulation. I Guyton A C and Jones C. E. (eds.), MTP I ternational Review of Science. *Cardiovascular Ph siology 1* 163 195, Butterworth, London University Park Press, Baltimore 1974

JOHNSON P C., K S. BURTON H HENRICH and U H NAIGI Effect of occlusion d ration on reactive hyperemia in sartorius muscle capillaries. *Amer J Physiol* 1976 230 715 719

LUNDVALL, J Tissue hyperosmolality as a mediator of asod latation and transcapillary fl id fl in exercising keletal muscle. *A t ph siol scand* 1972. Suppl. 379 1 142.

LUND ALL, J S. MELLA DER and H SPARKS, Myogenic response of resistance essel nd precapillary sphincters in skeletal muscle during exercise. *Acta ph siol. scand* 1967 70. 257 68

Mellander, S., a) *Control of capillary fluid transfer by precapillary and postcapillary vascular adjustments.* IXth World Conference of European Microcirculation Society. Antwerp July 1976. Karger Basel.

Mellander, S. b) *Static and dynamic components in myogenic autoregulation.* XIth International Congress of Angiology Tokyo. Sept. 1976.

Mellander, S. and S. Arvidsson, Possible "dynamic" component in the myogenic vascular response related to pulse pressure distension. *Acta physiol. scand.* 1974. 90. 283–285

Mellander, S. and B. Johansson, Control of resistance, exchange, and capacitance functions in the peripheral circulation. *Pharmacol. Rev.* 1968. 20. 117–196.

Mellander, S., J. Lundvall and P.-O. Grände, Evidence for a dynamic component in the myogenic control of blood vessel tone in vivo. *Acta physiol. scand.* 1976. 96. 40A–41A.

Mellander, S., B. Öberg and H. Odelram, Vascular adjustments to increased transmural pressure in cat and man with special reference to shifts in capillary fluid transfer. *Acta physiol. scand.* 1964. 61. 34–48.

Melsa, J. L. and D. G. Schultz, *Linear Control Systems.* International Student ed. McGraw Hill Book Company. 1969

Sigurdsson, S. B., B. Johansson and S. Mellander, Dynamic myogenic responses of the isolated rat portal vein to passive changes in length under isometric and isotonic conditions. *Acta physiol. scand.* 1976 96 17A

Sigurdsson, S. B., B. Johansson and S. Mellander, Rate-dependent myogenic response of vascular smooth muscle during imposed changes in length and force. *Acta physiol. scand.* 1977 99 183–189

Somlyo, A. P. and A. V. Somlyo, Vascular smooth muscle I. Normal structure, pathology, biochemistry and biophysics. *Pharmacol. Rev.* 1968. 20. 197–272.

Wiedeman, M. P. Patterns of arteriovenous pathways. In: *Handbook of Physiology* ed. by W. F. Hamilton and P. Dow. The Williams & Wilkins Co., Baltimore, 1963. Sect. 2, vol. 2, pp. 891–933

causes an additional pressure rise, etc.) The *in vitro* studies (Johansson and Mellander 1975 Sigurdsson *et al* 1976) strongly indicated that any extreme effects of such a mechanism will not ensue since there was an upper biological limit of the dynamic response at high rates of stretch. Furthermore the chemical metabolic control system acts as a brake on the myogenic control system, since intense vasoconstriction is counteracted by the attendant local accumulation of vasodilator metabolites (Berne 1964) The dynamic component in myogenic control might also as mentioned, favour the development of a damped oscillatory response of the vascular smooth muscle when transmural pressure is changed with consequent alternating periods of excitation and inhibition caused by alternation between repetitive positive and negative rate-dependent stimuli This implies an inherent negative feedback component in the myogenic control which efficiently might hinder extreme myogenic effector reactions.

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References

BAEZ, S., Z. LAIDLAW and L. R. ORKIN, Localization and measurement of microvascular and microcirculatory responses to venous pressure elevation in the rat. *Blood Vessels* 1974 *11* 260-276.

BASAR, E. and C. WEISS, Rate sensitivity of the mechanism of pressure induced change of vascular resistance. *Kybernetik* 1969 *5* 241 47

BAYLISS, W M On the local reactions of the arterial wall to changes in internal pressure *J Physiol.* (Lond) 1902. *28* 220-231

BERNE, R M *Regulation of coronary blood flow Physiol. Rev* 1964 *44* 1 29

BEVAN J A and B. LJUNG, Longitudinal propagation of myogenic activity in rabbit arteries and in the rat portal vein. *Acta physiol. scand.* 1974 *90* 703-715

FOLKOW B., Intravascular pressure as factor regulating the tone of the small vessels. *Acta physiol. scand.* 1949 *17* 289-310

FOLKOW B Transmural pressure and vascular tone—some aspects of an old controversy *Arch int Pharmacodyn.* 1962. *139* 455-469

FOLKOW B., Description of the myogenic hypothesis. *Circulat Res.* 1964 *15* S ppl. 1 279-285.

FRONEK, K. and B. W ZWEIFACH, Microvascular pressure distribution in skeletal muscle and the effect of vasodilation. *Amer J Physiol* 1975 *228* 791 796

GRÄNDE, P-O J JÄRHULT and S. MELLANDER, Method fo gravimetric registration of changes tissue volume. *Acta physiol scand* 1974 *91* 11 215

HADDY F J A. G RICHARDS, J L. ALDEN and M. B. VISSCHER, Small vein and artery pressures in normal and edematous extremities of dogs under local and general anaesthesia. *Amer J Physiol.* 1954 *176* 355-360

JÄRHULT J and S. MELLANDER, Autoregulation of capillary hydrostatic pressure in skeletal muscle during regional arterial hypo- and hypertension. *Acta physiol scand* 1974 *91* 32-41

JOHANSSON, B. and S. MELLANDER, Static and dynamic components in the vascular myogenic response to passive changes in length as revealed by electrical and mechanical recordings from the rat portal vein. *Circulat Res.* 1975 *36* 76-83.

JOHNSON, P C., (ed.) *Symposium on autoregulation of blood flow Circulat Res.* 1964 *15* Suppl 1 1 291

JOHNSON, P C., The microcirculation, and local and humoral control of the circulation. In Guyton A C. and Jones C. E. (eds.), MTP International Review of Science *Cardiovascular Physiology 1* 163-195, Butterworth, London University Park Press, Baltimore 1974

JOHNSON, P C., K. S. BURTON, H HENRICH and U HENRICH, Effect of occlusion duration on reactive hyperemia sartorius muscle capillaries. *Amer J Physiol.* 1976 *230.* 715-719

LUNDVALL, J Tissue hyperosmolality a a mediator of vasodilatation and transcapillary fluid flux in exercising skeletal muscle. *Acta physiol. scand* 1972. *8* ppl. 379 1 142.

LUNDVALL, J S. MELLANDER and H SPARKS, Myogenic response of resistance vessels and precapillary sphincters in skeletal muscle during exercise. *Acta physiol scand* 1967 *70* 257 268

[illegible], B., a) *Control of capillary fluid transfer by precapillary and postcapillary vascular adjustments.* [illegible] World Conference of European Microcirculation Society. Antwerp July 1976. Karger Basel.
[illegible], B. b) *Static and dynamic components in myogenic autoregulation.* XXth International Congress [illegible] Tokyo. Sept. 1976.
[illegible], B. and S. ARVIDSSON, Possible dynamic component in the myogenic vascular response to pulse pressure distension. *Acta physiol. scand.* 1974. 90. 293–293.
[illegible], B. and B. JOHANSSON, Control of resistance, exchange, and capacitance functions in the [illegible] circulation. *Pharmacol. Rev.* 1968. 20. 117 196.
[illegible], B. J. LUNDVALL and P.-O. GRANDE, Evidence for a dynamic component in the myogenic [illegible] of blood vessel tone in [illegible]. *Acta physiol. scand.* 1976. 96. 40A–41A.
[illegible], B., B. ÖBERG and H. ODELRAM, Vascular adjustments to increased transmural pressure in cat [illegible] with special reference to shifts in capillary fluid transfer. *Acta physiol. scand.* 1964. 61. 34–43.
[illegible] L. and D. G. SCHULTZ, *Linear Control Systems.* International Student ed. McGraw Hill Book [illegible] 1968.
[illegible], S. B., B. JOHANSSON and S. MELLANDER, Dynamic myogenic responses of the isolated rat [illegible] to [illegible] changes in length under isometric and isotonic conditions. *Acta physiol. scand.* [illegible] 17A.
[illegible], S. B., B. JOHANSSON and S. MELLANDER, Rate-dependent myogenic response of vascular [illegible] muscle during imposed changes in length and force. *Acta physiol. scand.* 1977. 99. 183–189.
[illegible] A. P. and A. V. SOMLYO, Vascular smooth muscle: I. Normal structure, pathology biochemistry [illegible] physics. *Pharmacol. Rev.* 1968. 20. 197–272.
[illegible], M. P. Patterns of arteriovenous pathways. In: *Handbook of Physiology* ed. by W. F. Hamilton [illegible] Dow. The Williams & Wilkins Co., Baltimore, 1963. Sect. 2, vol. [illegible], pp. 891–933.

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Characteristics of Aortic Baroreceptor C Fibres in the Rabbit

By

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Abstract

THORÉN P and J V JONES. *Characteristics of aortic baroreceptor C fibres in the rabbit* Acta physiol. scand 1977 99 448–456

The characteristics of 40 C-fibres a sing from the aortic arches of 15 normotensi e rabbits ha e been investigated. The conduction velocity of the f bres aried between 0.5–1 8 m/s (0.91 ± 0 05 mean ± S E). The activation threshold for all the f bres lay between 70–140 mmHg 32 medullated f bres from the same nimals had thresholds between 35 and 90 mmHg After correction for the time delay i conduction the firing i the C f bres occurs in early systole. Pressure response curves were constructed for 10 medullated and 18 C-fibres. The mea activity t 100 mmHg was, for the C-f bres 5 0 Hz and for the medullated fibres 34 Hz. At 130 mmHg the activity i the C-f bres was 13 Hz and in the medullated fibres 68 Hz Thus C-fibres from the aortic baroreceptor regions are ct vated at h gher pressures tha the medullated fibres and have lower discharge frequencies. Nor drenaline did not influence the pressure response curves of the C-f bres. It is concluded that the rterial baroreceptor C f bres may exert a eak tonic nfluence on the vasomoto centre at rmal pressures but re l kely to be of greater mportance when the arterial pressure rises acutely

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Aortic baroreceptors with medullated afferents have been studied extensively in many species *e g* dogs, rabbits and rats (Kircheim 1976) However the aortic nerve also contains non-medullated fibres. Electrical stimulation of these non-medullated afferents in the aortic and also in the carotid sinus nerves can evoke powerful depressor reflexes (Douglas and Ritchie 1956, Douglas Ritchie and Schaumann 1956) with different efferent patterns from the medullated fibres (Kardon, Peterson and Bishop 1975) Activity in non-medullated baroreceptor afferents was first recorded by Fidone and Sato (1969) However Landgren (195) described baroreceptor afferents in the sinus nerve with a low signal to noise ratio which were probably C-fibres. Coleridge *et al* (1973) also studied aortic C fibres. These C-fibres in the dog and cat had very high thresholds. Recently Thoren Saum and Brown 1976 have studied the characteristics of aortic C fibres using an *in vitro* aortic arch preparation in rats. The C-fibre receptors in the rat had higher thresholds than the medullated receptors. Furthermore, they had only a sparse discharge at normal pressure but could be markedly activated when the pressure was elevated.

On lea e from the University Department of Medical Cardiology Royal Infirmary Glasgow Scotland

This study was undertaken to examine the characteristics of C-fibres arising from the [illegible] arch of the rabbit. In comparison with the previous work in the rat, this study was [illegible] on an *in vivo* preparation in order to keep the sympathetic efferent fibres to the [illegible] arch intact and to avoid artificial perfusion media. In addition the characteristics of [illegible] C-fibre receptors were compared with those of medullated aortic baroreceptors from the [illegible] animal.

Methods

[illegible] were performed on rabbits (1.5–2.5 kg b.w.). The animals were anaesthetized with sodium [illegible] (Nembutal, 30 mg/kg b.w.) after premedication with diazepam (Valium 5 mg) [illegible]. Additional doses of 5–10 mg/kg Nembutal were given [illegible] intervals as required. A tracheal cannula [illegible] and the animal put on positive pressure ventilation with 100 [illegible] oxygen at an end tidal pressure of [illegible] cm H_2O. Muscle movements were prevented by means of gallamine triethiodide (Flaxedil), 10 mg/kg [illegible] injections. Rectal temperature was measured and maintained at 37–38 C by means of [illegible] lamp. The PO_2, PCO_2 and pH were measured at intervals and PCO_2 and pH were maintained [illegible] the normal range (30–40 mmHg and 7.30–7.45 respectively) by adjusting the tidal volume and by [illegible] small amounts of $NaHCO_3$ (1 meq/ml) as required.

[illegible] vagi, the aortic nerves, the sympathetic trunks and the carotid arteries were dissected free [illegible] the [illegible]. The thorax was opened by [illegible] bilateral transsternal incision and snares were placed around the descending aorta, close to the diaphragm, and around the inferior caval vein. Through a slit in the pericardium the [illegible] aorta was exposed thus allowing it to be briefly clamped with forceps for localization of receptors. The arterial blood pressure was measured with [illegible] Statham transducer (P23 DC) connected to [illegible] catheter [illegible] inserted at the arch of the aorta via one femoral artery. The arterial pressure was recorded on [illegible] polygraph (model 7 D) and on an ultraviolet (UV) light recorder (ABEM 5651). One femoral vein [illegible] also cannulated.

[illegible] bipolar electrocardiogram (ECG) was recorded with one electrode inserted in the left side of the chest [illegible] and the other electrode placed in the oesophagus behind the heart. The ECG was displayed on both [illegible] oscilloscope (Tektronix 502) and on the UV-recorder.

[illegible] of nerve traffic

[illegible] left aortic nerve (in 2 rabbits the right aortic nerve) was placed on [illegible] small plate. A pool was made [illegible] the surrounding muscle and skin and [illegible] was filled with mineral oil. The nerve sheath around the aortic [illegible] was removed by means of sharp forceps under [illegible] binocular dissection microscope and thin filaments [illegible]. The filaments were placed on [illegible] bipolar chloridized silver electrode and connected to [illegible] bipolar [illegible]. The output was displayed both on the oscilloscope and on the UV-recorder. The amplifier was [illegible] connected to [illegible] loudspeaker and to [illegible] ratemeter (time constant 0.4 [illegible]) equipped with [illegible] discriminator [illegible] could count either all spikes exceeding [illegible] preset level or only spikes with amplitudes between two [illegible] levels. The output of the ratemeter was recorded on the Grass polygraph. The activity of [illegible] receptors was calculated from the spike counter recordings but numerous spot checks of the actual [illegible] recorder tracings were made in each experiment to ascertain the accuracy of the spike counter readings.

[illegible] conduction velocities of the fibres could be established from the time delay between an electrical [illegible] (Grass S4) applied to the aortic nerve, close to its entrance into the thorax, and from the distance [illegible] the stimulating and recording electrodes. Whenever possible the total conduction time was measured by applying the stimulating electrode to the area in the aortic arch where the receptor could be [illegible] activated mechanically.

[illegible] procedures

[illegible] activity in all filaments was observed during electrical stimulation of the aortic nerve close to the [illegible]. Filaments showing evidence of aortic C-fibre activity were dissected further until single or few [illegible] remained active. All filaments showing evidence of C-fibre traffic were then tested by [illegible] shortlasting [illegible] of the descending aorta.

The localization of the receptors to the aortic arch was established by the response to occlusion of the descending aorta and the lack of response to brief occlusion of the ascending aorta In many cases the exact anatomical location of the receptor was obtained by gently exploring the aortic arch with a fine blunt probe. Receptor activity in single or few fibre preparations was observed with alterations in aortic pressure either by means of graded caval vein occlusion or by means of graded occlusion of the descending aorta.
The blood pressure/receptor response curve was also determined during i infusion of noradrenaline

Results

The study is based on recordings from 40 aortic C fibres and 32 aortic medullated fibres in 15 normotensive rabbits. The awake mean arterial blood pressure ($\pm$S.E.) measured directly i.a. in the ear artery (21 g needle) was $83 \pm 1\ 8$ mmHg. The conduction velocity for the C fibres varied from 0.5–1 8 m/s ($0\ 91 \pm 0\ 05$ mean $\pm$S E.)

Location of the receptors

15 of the 40 receptors were located in the aortic arch by gentle probing with fine forceps r These 15 receptors were located throughout the entire aortic arch. The other 25 receptors were localised to the aortic arch by the augmented receptor activity during occlusion of the descending aorta and the lack of receptor response or decrease in activity during occlusion of the ascending aorta It was also evident that the aortic nerve in the rabbit contains fibres from receptors which responded to occlusion of the ascending aorta meaning that these receptors were probably located in the heart This was confirmed in many cases by mechanical probing of the heart These cardiac C-fibres were not subjected to further study Furthermore a few C-fibres with a sparse discharge related to the respiratory cycle were also found.

Threshold for activation

The threshold mean arterial blood pressure for activation of each of the 40 receptors with non-medullated afferents is shown in Fig. 1 As a comparison the threshold values for all 32 medullated receptors are also shown Only 10 of the 40 C-fibre receptors had threshold levels lower than the mean pressure in the unanaesthetized animal (83 mmHg) while almost all the medullated fibres had threshold values lower than the mean ambient blood pressure In general there is little overlapping in the thresholds of the medullated and non-medullated fibres.

The receptor response to a graded change in arterial pressure

The receptor response to graded changes in aortic arch pressure was tested in 10 medullated and 18 C-fibres.

Fig. 2 shows an example of the activity in a single aortic C-fibre during a graded caval and aortic occlusion The receptor has a spontaneous discharge of about 1 imp/heart beat (A) at a mean arterial blood pressure (MABP) of 100 mmHg. During the caval occlusion the receptor stops firing at a mean pressure of 95 mmHg. During graded occlusion of the descending aorta the activity increases in parallel with the rise in arterial pressure up to a maximum value of more than 20 Hz. The firing initially displays a clear cardiac rhythmicity (B, C) but becomes continuous (D) at high pressure

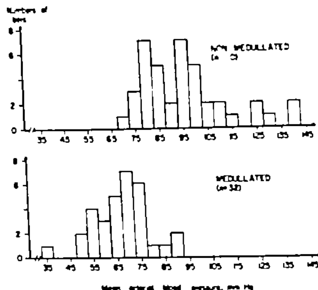


Fig. 1 The threshold blood pressure for activation of 40 non-medullated and 32 medullated fibres.

Fig. 3 shows the mean blood pressure/receptor response curve for 18 C-fibres and 10 medullated fibres during graded inferior vena caval and aortic occlusion. Many of the receptors with high thresholds could not be tested by graded occlusion of the descending aorta because the highest pressure then obtained during occlusion was just barely enough to activate these high threshold receptors. Furthermore it is highly probable that there exist aortic C-fibres with thresholds above the maximum blood pressure attained since many filaments contained C-fibres which could not be activated by aortic occlusion. These non-responsive fibres were not arising from chemoreceptors as they were not activated by asphyxia. The pressure response curve for the C-fibre discharge in Fig. 3 is thus not a correct reflection of the activity in the whole spectrum of aortic C-fibres because many high threshold receptors could not be included.

The firing pattern in the cardiac cycle

The firing pattern in relation to the cardiac cycle was examined for 9 receptors in which the total conduction time (50–130 m/s) from the receptor site to the recording electrode was known. By taking the time into account the corrected position of the receptor activation in the cardiac cycle could be determined. Fig. 4 is an example of this. This filament contains a C-fibre with a prominent upward deflection and 2 medullated fibres with more prominent downward deflections. The total conduction time (90 m/s) from the receptor site in the apex of the aorta was measured in C and by taking this delay into account the timing of the receptor activation in relation to the cardiac cycle could be established as indicated by the asterisks in A and B. The C-fibre is recorded as *firing* in late systole or in diastole but the receptor *activation* actually occurs in early systole in phase with the activation of the medullated fibres in the filament.

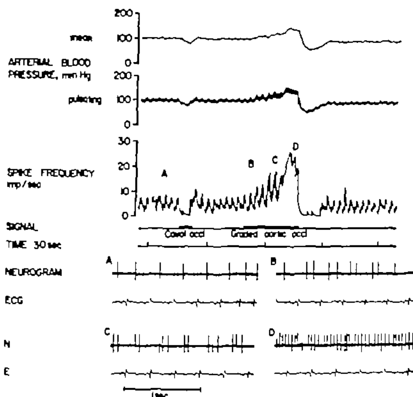


Fig. 2 Effects of graded occlusion of the inferior caval vein and the descending aorta on aortic blood pressure and spike frequency in a single aortic C-fibre. The letters in the spike frequency recordings correspond to the neurogram below

The effect of noradrenaline on the receptor discharge

The blood pressure/receptor response curve for 18 C-fibre receptors was compared during mechanical obstruction of the descending aorta and during injection of noradrenaline (NA) as shown in Fig. 5 15 of the receptors were studied when the NA was given in repeated bolus injections and 3 receptors during continuous infusion of NA (9 μg/min) for more than 5 min. The blood pressure rise consequent upon the infusion of NA was prevented by partial caval occlusion. Towards the end of the infusion the pressure response curves were determined by gradual release of this occlusion which resulted in a blood pressure rise. These two ways of administering NA gave similar results and there was no significant difference between the pressure response curves obtained during mechanical occlusion of the descending aorta or during administration of NA However it is likely that small differences would be missed in this comparison since the pulse pressure with caval occlusion or NA bolus injections was different from that present with aortic occlusion although the mean pressures could be kept the same.

Discussion

The existence of a large population of aortic baroreceptor C fibres (Landgren 1952, Landgren, Neil and Zotterman 1952, Fidone and Sato 1969 Coleridge *et al* 1973 Thoren Saum

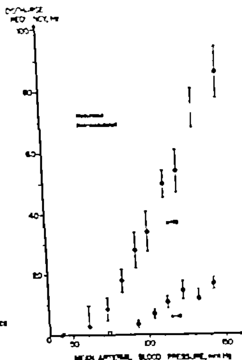


Fig. 3 Mean blood pressure/receptor response curves for 8 C-fibres and 10 medullated fibres.

and Brown 1976) was confirmed in this study The receptors were distributed throughout the whole aortic arch with their afferent connections in the aortic nerves and with conduction velocities of 0.5–1.8 m/s.

Receptor characteristics

In the control situation the receptors discharged with a low frequency or were silent. At the mean blood pressure (83 mmHg) of this group of animals only 25 per cent of the C-f bre receptors show evidence of activation in contrast to 95 per cent of the medullated f bres. Furthermore at that pressure the mean frequency of 18 tested C-f bres was 1 1 Hz in contrast to 18 Hz in 10 medullated fibres. There was little overlapping in thresholds for the medullated receptors and the C-f bre receptors. The C-fibres were *activated* in systole just as the medullated fibres and noradrenaline infusion did not seem to alter their pressure response curves significantly These data in an *in vivo* rabbit preparation agree with an earlier study by Thorén, Saum and Brown (1976) on an *in vitro* rat aortic arch preparation. The C-fibres arising from the rat aortic arch thus also seem to have higher thresholds and lower discharge frequencies than the medullated fibres. In the aortic arch preparation of the rat 2 different types of aortic C-fibres were described. One group with an irregular rather low frequency discharge and one group with a regular and more high frequency discharge. A similar analysis of the receptor discharge cannot be performed in the present study because it is not possible to alter the pressure in a square wave fashion in this *in vivo* preparation. However even in the present preparation there is marked variation in the receptor response to aortic

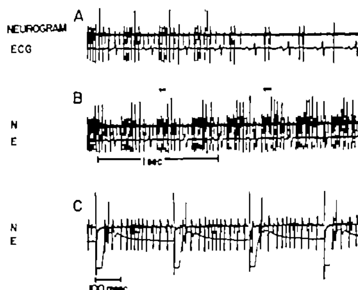


Fig 4 Firing pattern in relation to the cardiac cycle fo 1 C-f bre (prominent upward deflection) and medullated fibres (downward deflections) A During ca al occlusion. B During descending ortic occl sion. C. Total conduction time obtained by electrical stimulation over the receptor area Sign radicat corrected position of recept activation in cardiac cycle after total conduction t me as measured in is taken i to account.

occlusion Some receptors responded at moderate pressures with a high frequency and rel tively regular discharge (up to 42 Hz) while other receptors responded with only a lo frequency sporadic discharge even at high pressures. However whether the same differen between regular and irregular receptors as seen in the rat also exists in the rabbit cannot b established with the present preparation

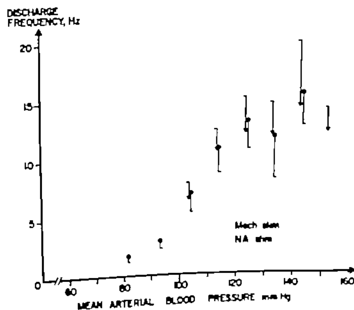


Fig 5 Blood pressure recepto response curves for 18 C-f bre obtained when blood pressure a varied by means of mechanic occlusion of the descending aort (c rcles) nd by means of injec tions or nfusion of noradrena line (triangles).

[Th]e present study further supports the classical study by Landgren (1952) where he [desc]ribed carotid sinus baroreceptor fibres with low signal to noise ratio which presumably [were] C-fibres. These receptors had a higher threshold and a lower discharge frequency [than] the medullated fibres arising from the carotid sinus. This study does not agree with the [rece]nt study on aortic C-fibres in the dog by Coleridge *et al.* (1973). They claimed that the [aort]ic C-fibres had very high and unphysiological thresholds. The difference in our results [mig]ht of course be due to species differences. In the studies of Coleridge *et al.* the fibres [were] evidently dissected mainly from the main vagus stem and not from the aortic nerve. [Thi]s at least a possibility that the aortic baroreceptors travelling in the vagus might ha e [some]what different characteristics. Furthermore the aortic fibres in their study were selected [acco]rding to their responses to drugs, while in the present study the receptors pressure [res]ponses were determined.

Many filaments ere examined containing C-f bres that could not be activated even by [ma]rked pressure increases in the aortic arch. Other C-fibres were also studied which had [su]ch high thresholds that the pressure could not be raised far enough to obtain other than [thr]eshold values for these receptors. Therefore the pressure response curves presented here [re]present those C-fibres with lower thresholds and are, therefore, not representative of the [w]hole group.

Mechanism of receptor activation

[Th]e aortic C-fibres respond to increased pressure in the aortic arch and they are activated [du]ring the systolic pressure wave as are aortic medullated receptors. It is therefore likely [th]at these receptors, as well as the medullated fibres, respond to stretch of the aortic wall. [Fur]thermore there is no data indicating that contraction of the smooth muscle elements in [th]e aortic wall with NA might influence the pressure response curves and thus that the [aort]ic C-fibres are coupled in series with the smooth muscle elements in the wall. This is [in] contrast to earlier data by Landgren, Neil and Zotterman (1952) and Thoren, Saum and [Br]own (1976). However in these expts. much stronger solutions of NA were used. Thus, [Lan]dgren *et al.* (1952) applied stocksolution of NA (1 mg/ml) on the sinus wall while [Th]oren *et al.* (1976) perfused the rat aortic arch with a solution containing 1 µg/ml which [is] considerably stronger concentration of NA than was used in the present study. It is [als]o possible that NA in very high doses may affect the pressure response curves but within [the] limitations of this study the data does not indicate that such an effect is of importance [wit]h physiological concentrations of NA.

[Re]flex effect and physiological role of aortic baroreceptor C-fibres

[Re]flex effects arising from aortic baroreceptor C-fibre activation during differentiated [ele]ctrical stimulation of the aortic nerve in the cat and the rabbit was demonstrated by Doug[las] and Ritchie (1956) and Douglas *et al.* (1956) who showed that the aortic C-f bres induced [po]werful depressor reflexes, t low stimulation frequencies. These reflexes were different [fro]m those arising from aortic baroreceptors with medullated fibres during electrical stimula[tio]n. The C-fibres had their main effect on the vagal outflow to the heart with only little [effe]ct on the sympathetic outflow (Kardon, Peterson and Bishop 1975).

The discharge in aortic C-fibre baroreceptors normally is sparse at the ambient pressures of these normotensive rabbits (range 70–90 mmHg) Thus the medullated fibres, because of their lower thresholds, may be the more important fibre group under normal conditions or when the blood pressure is reduced such as occurs with haemorrhage However during acute increases in blood pressure such as occurs in many everyday situations, C-fibre traffic will increase and have a greater inhibitory effect on the vasomotor centre. Possibly they might even surpass the medullated fibres in importance under these circumstances.

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References

Coleridge, H M J C. G Coleridge, A Dangel, C. Kidd J C. Luck and P Sleight Impulses in slowly conducting vagal fibres from afferent end ngs I the veins, atria and arteries of dogs and cats. *Circulat R s*. 1973 33 87 97

Douglas, W W and J M Ritchie, Cardiovascular reflexes produced by electrical excitation of non-medullated afferents in the vagus, carotid sinus and aortic nerves. *J Physiol.* (Lond.) 1956. *134* 167 178.

Douglas, W W J M Ritchie and W Schaumann Depressor reflexes from medullated and non-medullated f bres I the rabb ts aortic nerve. *J Physiol* (Lond.) *1956. 132. 187 198*

Fidone, S. J and A Sato, A study of chemoreceptor and baroreceptor A and C-fibres I the cat carotid nerve. *J Physiol* (Lond.) 1969 *205* 527 548

Kardon, M B. D F Peterson and V S. Bishop Reflex heart rate control via specific aortic nerve afferents in the rabbit. *Circulat Res* 1975 *37* 41–47

Kirchheim, H R., Systemic arterial baroreceptor reflexes. *Physiol. Rev* 1976. *56* 100–176.

Landgren, S On the excitation mechanism of the carotid baroreceptors. *A ta phy iol scand* 195_ 26 1 34

Landgren, S., E. Neil and Y Zotterman, The response of the carotid baroreceptors to the local adm nistra tion of drugs. *Acta physiol scand 1952. 25* 24–37

Thoren, P W R Saum nd A M Brown, Characteristics of aortic baroreceptors with non-medullated afferents in the rat *Circulat Res* 1977 In press.

Acta physiol. scand. 1977. 99. 457–461

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Alpha-Receptor Subsensitivity of Isolated Atria from Rats Following Physical Training or Repeated ACTH Injections

By

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Received 29 September 1976

Abstract

SILTOVUORI, A., R. TIRRI and M. N. E. HARRI. *Alpha-receptor subsensitivity of isolated atria from rats following physical training of repeated ACTH-injections.* Acta physiol. scand. 1977. 99. 457–461.

Decreased chronotropic and inotropic sensitivity of isolated atria to phenylephrine but not to isoprenaline was found in rats following repeated physical training by swimming or repeated ACTH-injections. These changes were induced within a week of subjection of animals to these treatments. It is concluded, that these effects are mediated by increased adrenergic activity which results in this subsensitivity of alpha-receptors.

Lowered sensitivity of cardiac α-receptors is induced in rats by cold-exposure (Harri *et al.* 1974) and by repeated injections of phenylephrine or isoprenaline (Tirri *et al.* 1976), as indicated by lowered atrial chronotropic response to an α-adrenergic drug phenylephrine. On the contrary, an increased sensitivity of cardiac α-receptors is found to be induced in frog and/or mammals by several conditions associated with low physiological activity of the animal. Such conditions are the inactivity of skeletal muscles (Szentiványi *et al.* 1970 a), propylthiouracyl treatment (Nakashima *et al.* 1971), hypothyroidism (Kunos *et al.* 1974), increased vagal influences on heart (Szentiványi *et al.* 1970 b) and low experimental temperature of the heart (Kunos and Szentiványi 1968, Buckley and Jordan 1970, Harri 1973). These effects are usually associated with lowered β-receptor sensitivity shown in inotropic and/or metabolic activity of the heart. Supersensitivity to the chronotropic effects of noradrenaline has been shown to appear in the dog heart in 1 to 3 days of treatment with reserpine (Fleming and Trendelenburg 1961, Westfall and Fleming 1968). This supersensitivity seemed to be nonspecific because it occurred also using calcium.

To find out a possible common physiological condition, we further studied the effect of physical stress conditions and repeated ACTH-injections on the adrenergic response of isolated atria.

Material and methods

A total of 112 adult male Sprague-Dawley rats, 200-300 g in weight were used in these expts. 48 of them were daily exercised by swimming for 20 min in water at 30°C for 6 or 20 days. A weight of 7 g was attached at the base of the tail. An equal number of intact rats served as the control group. A group of 14 animals received once daily s.c. injection of adrenocorticotropic hormone (3 IU/kg) for a week. An equal group of rats received injections of NaCl-solution.

The cumulative concentration-response curves for the response to isoprenaline (ISO) and phenylephrine (PHE) were determined using isolated atria at 37°C in Tyrode's solution. The number of animals in each group is presented in the graphs.

To measure the chronotropic response to PHE and ISO the animals were killed by decapitation. The atria were dissected from the hearts and suspended horizontally with a tension of 600 mg in 30 ml of Tyrode's solution gassed with 95 % O_2 – 5 % CO_2. The rate of spontaneous contractions was recorded on a Mingograph 4 jet recorder by means of a suction electrode as described earlier (Harri *et al.* 1974). For measuring the inotropic responses to PHE and ISO the cranial tip of the left atrium was suspended in Tyrode's solution by means of the pin with platinum electrodes on them. The caudal end was fixed into position with a hook connected to the force transducer on the Grass polygraph recorder. The contractions of the atrium were driven electrically from the stimulator at a steady rate of 120 beats per min. Square wave pulses with double threshold intensity (about 2.5 V) and a duration of 3 ms were used. The resting tension applied was 600 mg, otherwise the conditions were the same as in the measurement of chronotropic response. A single concentration-response curve was determined with each atrial preparation. The following drugs were used: isoprenaline hydrochloride (Isuprel, Winthrop), phenylephrine hydrochloride (Neo-synephrine, Winthrop) and adrenocorticotropic hormone (Isactid, Ferring).

Results

The graphs in Fig. 1 show that after 6 and 20 days of physical training by swimming, the positive chronotropic sensitivity to PHE was lowered. This can be seen in the concentration-response curves shifted to the right. Because the maximum responses were strongly reduced ($p < 0.01$), the EC_{50}-values did not differ significantly in any of the treated groups. The basic contraction frequency of rat atria was not changed after the swimming exercise as compared to the controls being 233 ± 6 and 226 ± 7 beats per min respectively.

The results of the inotropic responses to PHE and ISO are presented in Fig. 2. The graphs show that physical training by daily swimming for 6 days reduced the sensitivity to PHE but did not change the response to ISO. The results are thus similar to those in chronotropic response. *The basic contractile force was slightly but not significantly greater in rats after* training by swimming than in controls (176 ± 16 mg and 147 ± 14 mg, respectively, $p > 0.20$).

The results in Fig. 3 indicate the effects of repeated ACTH injections for 7 days on adrenergic atrial response. The chronotropic sensitivity to PHE was lowered after this treatment. The maximum response to PHE was reduced to about 50 % after ACTH-injections as compared to the control values and it was statistically significant ($p < 0.05$). The responses to ISO were the same in the control and experimental group. ACTH treatment did not cause any changes in the basic contraction frequency.

Discussion

The results of the present study are similar to those found in isolated atria from cold-exposed rats (Harri *et al.* 1974) and from rats injected chronically with PHE or ISO (Tirri *et al.* 1976). In all these studies the lowered chronotropic sensitivity to the α-adrenergic drug, PHE, was observed in isolated atria, but there was no change in response to the β-adrenergic

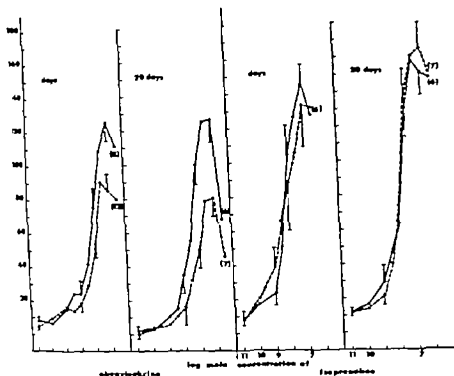


Fig. 1. Log concentration-response curves for the chronotropic responses to phenylephrine (A) and noradrenaline (B) in atria from control rats (continuous lines) and rats trained by daily swimming for 6 or 70 days (broken lines). The number of animals in each group is in brackets and vertical bars indicate ± S.E.

drug, ISO. It could be assumed that the enhanced adrenergic activity in all these experimental conditions was responsible for the subsensitivity of α-receptors observed (Harri *et al.* 1974, Tirri *et al.* 1976). The higher level of noradrenaline, as produced by cold, repeated injections of adrenergic drugs or physical training, could thus account for this lowered sensitivity of α-receptors. The subsensitivity induced by ACTH-injections may be a compensatory action of the stimulating effect of corticosteroids on the adrenergic action in heart muscle as shown in vitro by Kaumann (1972). On the other hand, the decreased sympathetic activity in rats resulting from decentralization or 6-hydroxydopamine treatment causes supersensitivity to noradrenaline but not to ISO (Trendelenburg 1963, Johansson 1973). This may also suggest a role of α-receptors in this supersensitivity. Kunos *et al.* (1973) have suggested that cardiac α- and β-receptors represent allosteric conformations of the same structure. They further suggest (Kunos *et al.* 1974) that changes in the thyroid hormone level result in an interconversion of myocardial α- and β-receptors. Our present and previous results indicate that the subsensitivity of α-receptors was not associated with a simultaneous increase in β-receptor response. This indicated that there was no interconversion of these receptors; thus giving support to Benfey (1975) who has also reported an opposing suggestion to the adrenoreceptor transformation hypothesis.

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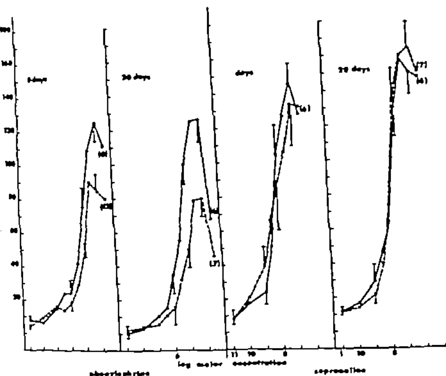


Fig 1 Log concentration–response curves for the chronotropic responses to phenylephrine (A) and isoprenaline (B) in atria from control rats (continuous lines) and rats trained by daily swimming for 6 or 28 days (broken lines) The number of animals in each group is in brackets and vertical bars indicate ± S.E.

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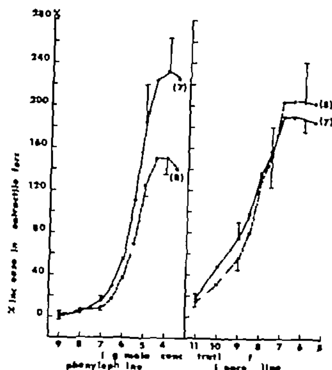
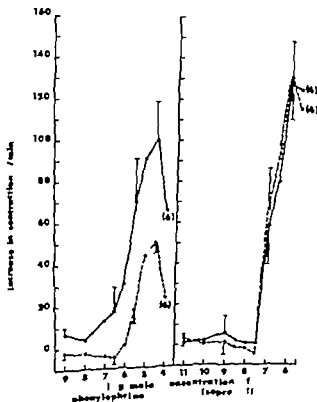


Fig. – Log concentration–response curves for the *inotropic responses* t phenyl-ephrine and isoprenaline in atria from control rats (continuous lines) nd rat trained by swimming for 6 days (broken lines). The umber of animals is in brackets and vertical bars indicate + or − S.E.



F g. 3 Log concentration–response curves for the chronotropic responses t phenyl-ephrine and isoprenaline atria from control rats (continuous lines) and rats injected with ACTH (3 IU/kg) for week (broken lines). The number of animals is in brackets and ertical bars indicate + or − S.E.

t of thyroxine as an inducer of changes in receptor sensitivity cannot be excluded.
n shown that cold-exposure (Heroux 1960) and β-adrenergic stimulation by ISO
et al. 1975) induce thyroid hormone secretion. Thus a change in sympathetic
ffects changes in thyroxine levels (Melander et al 1977). On the other hand, the
adrenergic action can be induced by an increase of thyroxine hormone level,
in hypothyroid or young animals (Swanson 1956, Lagerspetz et al. 1966). Thus,
ed adrenergic sensitivity even when caused by altered thyroxine level, could also
ed by a changed adrenergic action.

as supported by The Academy of Finland.

References

O Temperature dependence of phenoxybenzamine effects and the adrenoceptor transformation. Nature (Lond.) 1975. 256. 745–747

I. A. and C. C. Jordan, Temperature modulation of alpha- and beta-receptors in the isolated t. Brit. J. Pharmacol. 1970. 38. 394–398.

W and U Trendelenburg, The development of supersensitivity to norepinephrine after ent with reserpine. J. Pharmacol. exp. Ther. 1961. 133. 41–51.

K. E., Temperature dependent sensitivity of adrenoreceptors in the toad heart. Acta Pharmac.) 1973. 33 273–279

E., L. Meurman and R. Tirri, Changed chronotropic sensitivity to sympathomimetic amines d atria from rats following cold acclimation. Experientia (Basel) 1974 30 1041 1043.

Adjustment of the adrenal cortex and thyroid during cold acclimation. Fed. Proc. 1960. 19

B Effects of propranolol and a new cardioselective beta-blocker H 93/26, on responses of at atria to isoprenaline and noradrenaline. Europ. J. Pharmacol. 1973. 24 194–204

A. J. Potentiation of the effects of isoprenaline and noradrenaline by hydrocortisone in cat cle. Naunyn-Schmiedebergs Arch. exp. Path. Pharmak. 1972. 273. 134–153

and M. Szentiványi, Evidence favouring the existence of a single adrenergic receptor. Nature 968. 217 1077 1078.

M. S. Yong and M. Nickerson, Transformation of adrenergic receptors in the myocardium. ew Biol. 1973 241 119–120.

I. Vitrano-Kunos and M. Nickerson, Effect of thyroid state on adrenoceptor properties. Lond.) 1974 250 779–781.

K. Y. H. L. Autvanen and R. Tirri, The postnatal development of the metabolic response hine in mice. Ann. Med. exp. Fenn. 1966. 44 67–70

A., E. Nilsson and F. Sundler, Sympathetic activation of thyroid secretion. Endocrinology 194–199

A., E. Ranklev, F. Sundler and U. Westgren, Beta₂-adrenergic stimulation of thyroid secretion. Endocrinology 1975 97 332–336

M. K. Maeda, A. Seki and Y. Hagino, Effect of hypothyroid status on myocardial e to sympathomimetic drugs. Jap. J. Pharmacol. 1971 21 819–825

H. E. The effect of temperature on the potentiation of adrenalin by thyroxine in the albino rinology 1956 60 205–213

M. G. Kunos and A. Szentiványi Nagy, Modulator theory of adrenergic receptor mechanism f the dog hindlimb. Amer. J. Physiol. 1970 a. 218 869–875

M. G. Kunos and A. Juhász-Nagy, The adrenergic reactions of the coronaries in view of otheses of receptors. Acta physiol. Acad. hung. 1970 b. 37 427–428

A. Skurovuori and M. N. E. Harri, Alpha-receptor subsensitivity of isolated atria from rats g repeated injections of phenylephrine or isoprenaline. Experientia (Basel) 1976. 32 1283–1285

Trendelenburg, U. Supersensitivity and subsensitivity to sympathomimetic amines. Pharmacol. Rev. 225–276

D. P. and W. W. Fleming, Sensitivity changes in the dog heart to norepinephrine, calcium and pretreatment with reserpine. J. Pharmacol. exp. Ther. 1968. 159 98–106

Acta physiol. scand. 1977 99 462–466
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Effect of Hypoxia on Mitochondrial Mass and Cytochrome Concentrations in Rat Heart and Liver during Postnatal Development

By

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Abstract

KINNULA, V L. and I HASSINEN *Effect of hypoxia on mitochondrial mass and cytochr concentrations in rat heart and liver during postnatal development* Acta physiol sc 1977 99 462–466.

Cytochrome concentrations of rat heart and liver mitochondria were measured postnatally from new animals to young adults. Mitochondrial cytochrome aa_3 concentration increased shortly after birth in tissues, this concentration being in a newborn animal 0.149±0.077 nmol/mg protein liver and 0.1 0.082 mol/mg protein in heart The respective values from a two week old animal were 0.216±0.031 0.583±0.100 Postnatally cytochromes c+c and b increased markedly in the heart, but in the l er of born animals the cytochrome content was more close to the adult alues. The amount of mitochon protein increased after birth, too I a newborn animal the mitochondrial protei alues were 39 7 mg/g wet weight i liver nd 6.6±6.5 mg/g wet weight n heart. In adult nimals the respect e alues 71 5±4 8 and 80.7±13.1 The effect of postnatal hypoxia (10 O_2, 4 h and 48 h) on liver nd heart m chondrial cytochrome concentration and protein alues of newborn animals ere nvestigated. Du hypoxia the amount of mitochondrial protei remained bout at the level of a newborn nimal The p natal increase in the mitochondrial cytochrome concentration, hich was maller in the l er than in heart, was also inhibited by hypoxia

Ke ords: Heart, liver hypoxia, m tochondria cytochromes

In mammals, the formation of functional mitochondria is already almost complete dur intrauterine life (Hallman 1971). However in various rat tissues the activities of some mi chondrial enzyme systems, such as cytochrome oxidase, the succinate oxidase syst succinate dehydrogenase and succinate-cytochrome c reductase are low in the fetus and ne born as compared to the adult (Hallman 1971 Lang 1965 Jakovcic *et al* 1971 Gregson Williams 1969 Levy and Toury 1970) Also morphological changes in mitochondria oc postnatally Fetal mitochondria at term are large as compared with those from mature tis (Jakovcic *et al* 1971). In addition it has been observed that a disproportionate increase the mitochondrial mass occurs postnatally compared with the overall growth of the org parenchyma during the same period at least in the liver (Lang and Herbener 1972)

he synthesis of many components of the mitochondrial respiratory chain is accelerated ing the perinatal period (Jakovcic *et al.* 1971). The concentration of cytochromes increases her shortly after birth in different rat tissues (Hallman *et al.* 1972, Chepelinsky and iguez de Lores Arnaiz 1970, Linková *et al.* 1974). Factors that control these changes more or less unknown. To our knowledge, only one previous report exists on the effects oxygen on mitochondrial development postnatally and rather short experimental periods e used (Hallman 1971). The purpose of the present study was to investigate the role oxygen in regulation mitochondrial population and cytochrome content after birth and esign experimental conditions to allow longer observation periods.

Material and methods

ar rats were used and the age of the animals varied from newborn to 2 week old animals. In addition, t animals (age 90–100 days) of both sexes were used. Newborn rats were separated from their mothers placed in plastic hypoxia chamber in an atmosphere of 10 % O_2 in nitrogen at 101 kPa (760 mmHg) periods of 24 h and 48 h. Temperature was maintained at 21 C with relative humidity nearly 100 %. To keep the CO_2 concentration as low as possible, soda-lime CO_2 absorbers were incorporated in the mber. The gas flow was kept about 8 litres/h.

r control groups were maintained simultaneously for 24 h and 48 h in normal atmosphere, those with mother and those separated from their mother. The animals were decapitated and all homogenates pt those from the adults were prepared from pooled tissue of the animals to obtain sufficient amounts erial for analyses. Liver mitochondria were isolated in medium containing 0.27 M sucrose, 1.0 mM TA and 5.0 mM Tris-Cl (pH 7.4). The mitochondria were sedimented at 8 200 *g* by typical Schneider edure (Kuusela 1976). Heart mitochondria were isolated with Bacillus subtilis alkalase (obtained Novo Industri A/S, Denmark) according to Tyler and Gonze (1967). The isolation medium was 5 M mannitol, 0.075 M sucrose and 0.05 mM EDTA (pH 7.4). One washing of the fractions was carried The cytochrome assays were performed using a split beam spectrophotometer with 2 nm bandwidth in Elmer Double Beam 124) essentially according to Hallman *et al.* (1972). The concentration of mito- drial protein in the tissue was calculated from the cytochrome aa_3 content in the total homogenate and isolated mitochondria (Schollmeyer and Klingenberg 1962). Protein was determined by the method of ry *et al.* (1951).

Results

verage weight of a newborn animal was 5.24 ± 0.4 g. Attempts to induce nursing of the born rats by the mother after prolonged adaptation period and parturition in the xic atmosphere were not successful. As result, the newborn had to be deprived of the her and kept without food. To test the effect of fasting, one group of animals was main- ed under normal conditions separated from their mother. During the hypoxic period of 0–30% of the animals died depending on the litter; the respective value of the fasting rols was 0–10 %.

ytochrome concentration in the heart is markedly greater than in the liver. In both es especially cytochrome aa_3 concentration increased after birth. These results are nted in Fig. 1. Most prominent increase occurred during the first two days after birth. lar increase was also observed in cytochromes c + c and b postnatally. Both total and chondrial protein concentration increased after birth in both tissues (Table I). In a new- animal the content of mitochondrial protein in the tissue was 39.7 ± 3.6 mg/g wet weight he liver and 26.6 ± 6.5 mg/g wet weight in the heart. In adult animals the respective values e 71.5 ± 4.8 and 80.7 ± 13.1.

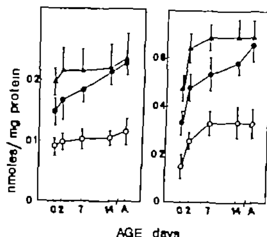


Fig. 1 Mitochondrial cytochrome concentrations in rat heart and liver during postnatal development. Values are means of 6–10 determinations. ●—● cytochrome aa_3, ○—○ cytochrome b and ▲—▲ cytochromes c + c. Left: liver mitochondria. Right: heart mitochondria. The vertical bars represent S.D.

Mitochondrial protein in the tissues in the hypoxic groups remained significantly lower than in the control group or in the starved group (Table I). Concerning the mitochondrial cytochrome content from one and two-day-old animals, it could be noticed that in both tissues greatest concentrations were obtained in the control group. After the hypoxic period of 24 h the cytochrome content in both tissues was lower than in the fed or fasting control groups. These differences remained qualitatively the same after the 48-hour observation period, the inhibition of the cytochrome induction was largest in the hypoxic groups, but an inhibition of a smaller extent occurred also in the fasting control group (Fig. 2).

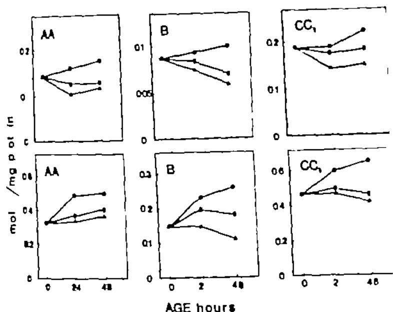
Discussion

Since the purpose of this study was to investigate the role of atmospheric oxygen in regulation of mitochondrial cytochrome content and protein values after birth, optimal and most

TABLE I. Total and mitochondrial protein concentration in heart and liver. Values are means ± S.D. from 4–8 determinations.

Age	Liver		Heart	
	Total mg/g wet wt.	Mitochondrial mg/g tissue wet wt.	Total mg/g wet wt.	Mitochondrial mg/g tissue wet wt.
Newborn	131.2 ± 23.9	39.7 ± 3.6	109.4 ± 22.0	26.6 ± 6.5
1 day	139.3 ± 19.0	36.6 ± 6.0	99.3 ± 10.0	23.5 + 6.2
2 days	137.7 ± 21.3	49.9 + 15.9	105.3 ± 22.1	31.2 + 5.6
7 days	160.5 ± 7.8	55.6 ± 12.1	121.3 ± 12.1	38.5 + 7.5
14 days	12.7 ± 26.5	73.3 ± 13.3	124.1 ± 12.1	49.7 + 8.2
Adult	307.7 ± 21.5	71.5 ± 4.8	173.7 ± 15.3	80.7 + 13.1
1 day (starved)	145.5 ± 15.0	36.8 ± 7.2	94.3 ± 7.0	26.9 ± 4.3
1 day (hypoxic)	136.7 ± 12.0	26.3 ± 6.8	104.6 ± 17.9	[illegible]2.3 ± 5.6
2 days (starved)	163.2 ± 14.9	44.6 + 12.4	122.6 ± 27.8	31.3 ± 6.7
2 days (hypoxic)	155.8 ± 29.8	4.9 + 8.4[a]	138.6 + 35.7	20.5 ± 4.7[b]

[a] Significantly lower value ($p < 0.01$) than in starved animals and in control animals of same age
[b] Significantly lower value ($p < 0.05$) than in starved animals and in control animals of same age



1. Mitochondrial cytochrome concentrations in rat heart (lower pictures) and liver (upper pictures) ...ed (●—●), starved (■—■) and hypoxic (▲—▲) rats during the first tw days after birth. Values ...ns of 6–10 determinations

...ble experimental conditions would have been reached if the animals had been born in ...poxia chamber. In Wistar rats this did not meet with success, and simply because of ...tion of the newborn by the mother. Perhaps some other more composed strain might ...been more suitable. I this case the newborn animals were in normal atmosphere for ...e time, though for as short a time as possible.

...e results show that protein concentration both in the total homogenate and in isolated ...chondria increased in both control and starved animal after birth. Because mito...drial protein alues after the hypoxic periods remained significantly lower than in ...ed animals, it seems that oxygen lack had primarily an effect on the development of ...chondrial proteins after birth. By comparing the results of the determination of cyto...mes and mitochondrial protein concentration in the tissues, an interesting observation ...be made, viz. the mitochondrial protein concentration was not influenced by fasting as ... the cytochrome concentration during the postnatal period. However a significant ...rease in the concentration of mitochondrial protein concentration in the tissue was ...nd. Dehydration of the fasting animals caused a small degree of increase in the tissue ...tein concentration, as is evident in Table I. This effect, however does not interfere with ...interpretation of the results, i.e. this effect does not influence the difference between the ...ting and hypoxic-fasting animals.

When newborn rats have been maintained in hypoxia for 12 h (10–11% O_2) no increase ...cytochromes c c or the rate of mitochondrial amino acid incorporation has been found ...lfman 1971), though mitochondrial protein and cytochrome content increases postna...ly in normal conditions (Halfman 1971 Halfman et al. 1972, Chepelinsky and Rodriguez

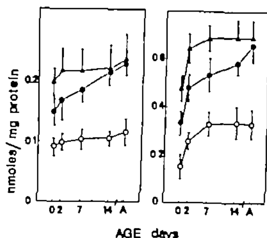


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Mitochondrial protein in the tissues in the hypoxic groups remained significantly lower than in the control group or in the starved group (Table I). Concerning the mitochondrial cytochrome content from one and two-day-old animals, it could be noticed that in both tissues greatest concentrations were obtained in the control group. After the hypoxic period of 24 h the cytochrome content in both tissues was lower than in the fed or fasting control groups. These differences remained qualitatively the same after the 48-hour observation period, the inhibition of the cytochrome induction was largest in the hypoxic groups, but an inhibition of a smaller extent occurred also in the fasting control group (Fig. 2).

Discussion

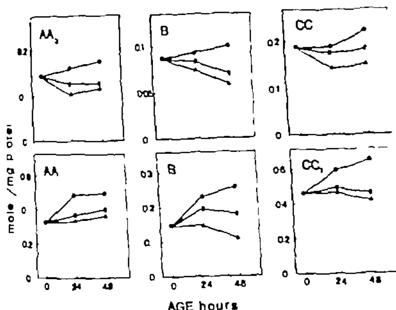
Since the purpose of this study was to investigate the role of atmospheric oxygen in regulation of mitochondrial cytochrome content and protein values after birth optimal and most

TABLE I Total and mitochondrial protein concentration in heart and liver. Values are means ± S.D. from 4–8 determinations

Age	Liver		Heart	
	Total mg/g wet wt.	Mitochondrial mg/g tissue wet wt.	Total mg/g wet wt.	Mitochondrial mg/g tissue wet wt.
Newborn	131.. ± ..3 9	39 7 + 3 6	109 4 + 2 0	6 6 ± 6 5
1 day	139 3 ± 19 0	36 6 + 6 0	99 3 + 10 0	23 5 + 6
2 days	137 7 ± 21 3	49 9 ± 15 9	105 3 + 1	31.. + 5 6
7 days	160.5 ± 7.8	55 6 + 1 1	121 3 + 12.1	38.5 7 5
14 days	12.7 ± 26 5	73 3 13 3	124 1 + 1 1	49 7 8
Adult	307 7 ± 21.5	71 5 + 4 8	173 7 _ 15 3	80 7 + 13 1
1 day (starved)	145.5 ± 15 0	36 8 + 7	94 3 7.0	6 9 4 3
1 day (hypoxic)	136.7 ± 1 0	6 3 + 6 8	104 6 + 17 9	.. 1 5 6
2 days (starved)	163 2 ± 14 9	44 6 + 1 4	1.. 6 7 8	31 3 6 7
2 days (hypoxic)	155.8 ± 29 8	4 9 8 4[a]	138 6 35 7	20 5 4 7[b]

[a] Significantly lower value ($p < 0.01$) than in starved animals and in control animal of same age

[b] Significantly lower value ($p < 0.05$) than in starved animals and control animal of same age



Mitochondrial cytochrome concentrations in rat heart (lower pictures) and liver (upper pictures) of (●—●), starved (■—■) and hypoxic (▲—▲) rats during the first tw day after birth. Values ns of 6-10 determinations

i experimental conditions would have been reached if the animals had been born in xia chamber. In Wistar rats this did not meet with success, and simply because of n of the newborn by the mother. Perhaps some other more composed strain might een more suitable. In this case the newborn animals were in normal atmosphere for time, though for as short a time as possible.

results show that protein concentration both in the total homogenate and in isolated ondria increased in both control and starved animals after birth. Because mito-rial protein alues after the hypoxic periods remained significantly lower than in d animals, it seems that oxygen lack had primarily an effect on the development of hondral proteins after birth. By comparing the results of the determination of cyto-s and mitochondrial protein concentration in the tissues, an interesting observation e made, iz. the mitochondrial protein concentration was not influenced by fasting as the cytochrome concentration during the postnatal period. However a significant ase in the concentration of mitochondrial protein concentration in the tissue was d. Dehydration of the fasting animals caused small degree of increase in the tissue n concentration, as is evident in T ble I. This effect, however does not interfere with nterpretation of the results, i.e. this effect does not influence the difference between the ng and hypoxic-fasting animals.

'hen newborn rats have been maintained in hypoxia for 12 h (10-11 % O_2) no increase ytochromes c or the rate of mitochondrial amino acid incorporation has been found llman 1971), though mitochondrial protein and cytochrome content increases postna-y in normal conditions (Hallman 1971 Hallman et al 1972, Chepelinsky and Rodriguez

de Lores Arnaiz 1970 Lisková *et al* 1974) In the studies of Hallman (1971) as well in the present study oxygen lack inhibited the development of mitochondrial cytochrom after birth However the present results emphasize that not only the cytochrome but al the total mitochondrial mass as reflected by the mitochondrial protein are regulated by t ambient oxygen tension in mammals.

These results are in full agreement with those obtained from lower animals or mammalı fibroblasts in low oxygen tension (Biggs and Linnane 1963 Wimpenny *et al* 1963 Whi 1964 Pious 1970) In addition, Park *et al* (1973) with cyanotic dogs and Kinnula (197 with hypoxic rats have obtained decreased concentration of mitochondrial cytochromes. O the basis of this study it seems probable that during postnatal development environment oxygen tension is an important factor that regulates the synthesis of mitochondrial protein after birth

References

BIGGS, D R. and A. W LINNANE, The effect of oxygen on the composition and orga ısation of the electro transport system of yeast. *Biochim. biophys. Acta* (Amst.) 1963 *78* 785–788.

CHEPELINSKY A. B and G RODRIGUEZ DE LORES ARNAIZ, Levels of cytochromes ı rat-brain mitochondr during postnatal development. *Biochim. biophys Acta* (Amst) 1970. *197* 321 3_3

GREGSON, N A. and P L WILLIAMS, A comparative study of brain and liver mitochondria from new-bor and adult rats. *J Neurochem.* 1969 *16* 617–6_6.

HALLMAN, M Changes in mitochondrial respiratory chain proteins during perinatal development E dence of the importance of environmental o ygen tension *Biochim. bioph Act* (Amst) 1971 *25* 360–372.

HALLMAN M P MÄENPÄÄ and I HASSINEN, Levels of cytochromes n heart, liver kidney and bra in th developi g rat. *Experientia* (Basel) 1972 *28* 1408–1410.

JAKOVCIC, S. J HADDOCK, G S. GETZ, M RABINOWITZ nd M SWIFT Mitochondrial development in liver of foetal and ew born rats. *Biochem J* 1971 *121* 341 347

KINNULA, V L., Mitochondrial cytochrome concentrations rat heart and l ver as a consequence of different hypoxic periods. *Act physiol. scand* 1976. *96* 417–4 1

LANG, C. A., Respiratory enzymes in the heart and liver of the prenatal and postnatal rat. *Biochem. J* 1965. *95* 365–371

LANG, C. A. and G H HERBENER, Quantitat ve comparison of the mitochondrial populations in the livers of newborn and weanling rats. *Developmental Biology* 1972. *29* 176–182.

LEVY M and R TOU Y Etude de l evolution des activites des enzymes mitochondriaux de l'hepatocyte au cours du developpement du rat. *Biochim bioph Acta* (Amst.) 1970. *216* 318–3 7

LISKOVÁ, Z. A STRUNECKÁ and Z. DRAHOTA, The cytochrome and phospholipid content of the mito-chondrial membrane of the neonatal and adult rat kidney *Physiol bohemoslov* 1974 *23* 221 _8.

LOWRY O H N J ROSEBROUGH, A. L. FARR a d R. J RANDALL, Protei measurement w th the Fol Phenol reagent. *J biol. Chem.* 1951 *193* 65–275

PARK, C. D L MELA, R. WHARTON J REILLY P FISHMAN and E. ABERDEEN, C rdiac mitochondria activity in acute and chronic cyanosis *J Surg Re* 1973 *14* 139–146.

PIOUS, D A *Induction of cytochromes* huma cells by o ygen *Proc nat Acad Sc* (Wash.) 1970. *65* 1001 1008

SCHOLLMAYER, P and M KLINGENBERG, Über den Cytochrom-Gehalt tierischer Gewebe. *Biochem. Z* 1962. *335* 426–439

TYLER, D D and J GONZE, The preparation of heart mitochondria from laboratory an mals I *Meth in En.. X* (eds. R. W Estabrook and M E. Pullman) Academic Press, New York and London 1967 75–77

WHITE, D C., Differential synthesis of fi primary electron transport dehydrogenases n *Hemophilus para-influenzae J biol. Chem.* 1964. *239* 2055–2060.

WIMPENNY J W T M RANLETT and C. T GRAY Repression and derepression of cytochrome biosy the- sis in *Escherichia coli*, *Biochim. biophys. Acta* (Amst.) 1963 [illegible]

Acta physiol. scand. 1977 99 467–475

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The Effect of 6-Aminonicotinamide Blockade of the Pentose Phosphate Pathway on Catecholamines in the Rat Adrenal Medulla, Superior Cervical Ganglion, Hypothalamus and Synaptosome Fractions

By

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Abstract

...SON, S.-E., J. GRIPENBERG and M. HÄRKÖNEN. *The effect of 6-aminonicotinamide blockade of the pentose phosphate pathway on catecholamines in the rat adrenal medulla, superior cervical ganglion, hypothalamus and synaptosome fractions.* Acta physiol. scand. 1977 99 467–475

...effect on tissue catecholamines of blockade of the pentose phosphate pathway with 6-aminonicotinamide (6-AN) was studied in the rat. 6-AN at 35–50 mg kg^{-1} permanently lowered the adrenaline content of the adrenal gland to less than 10 % of control values and caused 50 % loss of noradrenaline, ...such... When the amine turnover rate was increased by a preceding period of drum stress, 6-AN also ...ly depressed noradrenaline in the gland. 6-AN was without significant effect on the noradrenaline ...ation in heart tissue, hypothalamus and superior cervical ganglion and did not affect the uptake or ...of catecholamines in vitro. The possibility is discussed that 6-AN interferes with the biosynthesis of ...holamines, ...as it blocks the pentose phosphate pathway by decreasing the supply of reducing equiv... in the form of NADPH which are necessary for the tetrahydropteridine cofactors of tyrosine ...oxylase.

...words: 6-Aminonicotinamide, pentose phosphate pathway, amine uptake, storage, release, adrenals, ...

...osine hydroxylase, the rate limiting enzyme in catecholamine biosynthesis (Levitt *et al.* ...5) requires tetrahydropteridines as cofactors (Nagatsu, Levitt and Udenfriend 1964) ...se are kept in reduced state in a reaction involving NADPH. As the formation of ...DPH in neural tissue takes place largely by the pentose phosphate pathway (Härkönen ...Kauffman 1974), glucose metabolism via this pathway may be of importance for the ...intenance of tyrosine hydroxylase activity. This possibility could be tested with 6-amino...otinamide (6-AN). In the rat, administration of 6-AN results in the formation of the ...mino analogue of NADP which, by inhibiting 6-phosphogluconate dehydrogenase, ...cks the pentose phosphate pathway (Herken, Lange and Kolbe 1969). If this pathway is

of importance for tyrosine hydroxylase, the 6-AN-induced block would result in loss of tissue catecholamines It has indeed been shown that 6-AN lowers the adrenal catechol-amine content, but this effect was thought to be due to reduced granular uptake of catecholamines (Schacht Schultz and Senft 1966)

In the present paper the effects of 6-AN on the uptake, storage and release of catecholamines in various neural tissues of the rat have been investigated. The results to be reported indicate that 6-AN *has no effect on noradrenaline in the superior cervical ganglia* and hypothalamus but that adrenal catecholamines are lowered by 6-AN through a mechanism which partially depends on the turnover rate of the catecholamines.

Methods

Adult Sprague-Dawley rats f both sexes weighing 180–220 g were used When the effects of 6-AN on tissue catecholamine levels were studied, animals were injected with 6-AN dur g the morning hou and kept under observation for up to 24 h n laboratory surroundings with free access t water b without food 6-AN was administered intraperitoneally in saline, while controls received a sal ne injectio Tissue specimens were taken in urethane anaesthesia quenched l liquid nitrogen and tored on CO_2-i before determinatio of catecholamines. The hypothalamus was dissected accord g to Glow nski ar Iversen (1966). The weights *of the hypothalami in the control and 6-AN-treated animals did not diff* (60.0 ± 1.5 and 59 5 ± 3 0 mg, respectively) The tria of the heart were dissected, soaked in saline as dried by blotting before they were frozen. The adrenal glands were freed from periadrenal fat and th superior cervical ga glia were dissected free from the adhering connective t ssue The mean weight of a drenal gland in a control nimal was 20 ± 1 6 mg. Twelve hours after injection of 6-AN (35–50 mg kg^{-1} the weight of the adrenals had ncreased to 112 of the control value, but the d fference was not statist cally significant (p 0 1 nd 0 4 2 expts., respectively).

Drum stress. Rats were exposed to drum tress by placi g the animals in 40 cm wide drum with diameter of 56 cm and which rotated at 5 rpm. The drum stress started at 19 00 and lasted for 14 h At the end a few rats were killed for evaluation of the mmediate effect on tissue catecholamines. The remaining animals were divided to 2 groups one received 6-AN at 35 mg kg^{-} i.p the other saline After the njection, the nimals were llowed to recover w th free access t water but without food. The replenishment of tissue catecholamines was followed f 24 h.

Catecholamine uptak and effl xperiment Adrenal lices, 360 μm thick were prepared w th a M Ilwai tissue chopper The slices were ncubated a physiological electrolyte medium (see below) buffered to pH 7 4 w th carbonat buffer Pinched-off nerve endi gs (synaptosomes) were isolated from whole brain homogenates by density-gradient centrifugation (Ficoll-sucrose gradient) and incubated in a Tris-HCl buffered medium t be described elsewhere (n preparation)

In the *amine uptake expts ynaptosomes were preincubated f 5 m* at 37°C. Thereafter H-nor adrenaline at a specific activity of 0.64 C $mmol^{-}$ was added to the incubation system consist g of l ml incubation med um supplemented w th 0.2 mg ml ascorbic cid, 10^{-4} mol l nialamide and 1 mg ml^{-} synaptosomal protein (proteins determ ned according to Lowry *et al* 1951) The f nal amine concentration was $1.5 \times 10^{-}$ mol l^{-} Uptake was terminated by vacuum f ltration through Millipore filter (pore size 0.45 μm diameter 25 mm) and the f lter and synaptosomes were washed th 2 cha ges of fresh ncubation medium (3 + 7 ml) The filters were semi-dried n ai dissolved in 10 ml of Bray liquid scintillation medium (1960), *and counted* in a LKB-Wallac 81000 liquid scint llation counter D ring the first 5 mi t 37 C the uptake was approximately linear and between 0 3 nd 1 6 mg ml^{-1} was proportional t the protein concentration in the medium. Amine uptake was expressed mol g^{-} protei nd w corrected for zero-time uptake obtained by nterrupting the ptak process by f ltration and washing immediately after add tion of the radioactive substrat t *the system*

In some expts., the efflux of amines was followed. Adrenal slices were preloaded w th H-adrenaline (specific activity 14 Ci $mmol^{-}$ final concentration, 7 10^{-} mol l^{-1}) for 30 mi at 37°C After 3 washes, the slices were reincubated with or without added 6-AN At nous times, samples f the incubation medium were transferred to bottles containing 10 ml of Bray's *solution and counted The count g* efficiency determined with H-hexadecane as internal tandard was 4

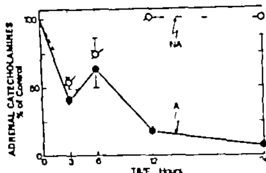


Fig. 1 Effect of 6-AN on adrenal catecholamines once 6-AN was injected i.p. at 35 mg kg^{-1} Values are expressed as per cent of the controls killed at appropriate time intervals after the injection. Each point is the mean ± S.E. for 4–8 rats. A = adrenaline, NA = noradrenaline.

Determination of 6-phosphogluconate (6-PG) 6-Phosphogluconate was measured fluorometrically by direct enzymatic assay (Kauffman *et al.* 1969).

Determination of glucose use The adrenal slices, contained in a small net-basket of stainless steel wire, were incubated in 50-ml centrifuge tubes in the incubation medium containing 5.5 mmol l^{-1} glucose for 1 min. Thereafter the basket was transferred to another centrifuge tube containing glucose-free incubation medium. After a rapid rinse in this medium the basket was pulled up to hang in the tube out of contact with the incubation solution. The outlet of the gas tubing was closed and O_2–CO_2 shut off. At zero-time, 1 min and 20 min some of the slices were rapidly sampled, frozen in liquid N_2 and assayed for glucose (Lowry, Passonneau and Lowry 1969).

Determination of catecholamines Tissue samples were homogenized and extracted according to Anton and Sayre (1968) in 0.2 mol l^{-1} phosphate buffer pH 7.8, which contained 24 mg p-chloromercuriphenylsulphonic acid per g tissue and 10 mg ml^{-1} EDTA. The fluorometric determination of adrenaline and noradrenaline was by the trihydroxyindole method (Anton and Sayre 1968, Weil-Malherbe 1968). The amounts of noradrenaline and adrenaline were calculated from fluorescence readings at wavelengths 400/485 and 430/510 nm, respectively (uncorrected instrumental readings) using an Aminco-Bowman spectrophotofluorometer. The recovery from internal standards was 75.1 ± 3.3 and 93.0 ± 2.2 for noradrenaline and adrenaline, respectively. Values were expressed in nmol or µmol per g tissue or per pair of ganglion and refer to free bases corrected for blank readings and recoveries.

The significance of differences between means was determined using the t-test.

Solutions and drugs The incubation medium had the following millimolar composition: NaCl, 136; KCl, 5.6; $MgCl_2$, 1.3; $CaCl_2$, 2.2; PO_4^{3-}, 1.3; glucose, 5.5 and bovine serum albumin at 1 mg ml^{-1}. In the experiments with synaptosomes the medium was buffered to pH 7.4 with 20 mmol l^{-1} Tris-HCl buffer, whereas in the experiments with adrenal slices carbonate buffer was used (16.2 mmol l^{-1} $NaHCO_3$, the medium being continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2). H-noradrenaline and H-adrenaline (specific activities of 0.64 Ci mmol^{-1} and 14 Ci mmol^{-1}, respectively) were obtained from the New England Nuclear Corporation. 6-AN was obtained from the Sigma Chemical Company.

Results

Effect of 6-AN on adrenal catecholamines The concentration of noradrenaline and adrenaline in the adrenal glands of control animals was 0.588 ± 0.053 and 3.36 ± 0.33 µmol g^{-1}, respectively (means ± S.E. of 31 animals). 6-AN administered i.p. at 35–50 mg kg^{-1} practically depleted the adrenals of adrenaline in 12 h, whereas noradrenaline was only transiently lowered. 3 h after the injection the noradrenaline content was reduced to 55% of the control level while adrenaline stores were even lower (Fig. 1). After this initial decline, the noradrenaline content was gradually restored, being equal to the control within 1 h. Adrenaline was replenished only to a limited extent; 6 h after the injection, a second phase of depletion started

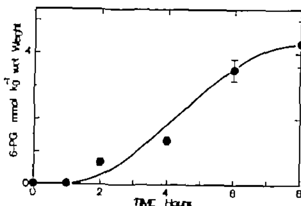


Fig. 2. Accumulation of 6-phosphogluconate (6-PG) in the adrenal medulla after in vivo treatment with 6-AN. 6-AN was injected i.p. at 50 mg kg⁻¹. Each point represents the mean ±S.E. for 3 animals. The zero-time 6-PG concentration was 16.5 μmol kg⁻¹.

which brought adrenaline levels down to 18 and 7 % of the control values at 12 and 24 h, respectively. In the controls the adrenaline: noradrenaline ratio was 6:1. This ratio was reversed after treatment with 6-AN.

The first phase of 6-AN-induced amine depletion in the adrenals took place when the pentose phosphate pathway was still operative, whereas the second phase of adrenaline depletion did not occur until metabolism via the pathway was blocked. This was shown by following the accumulation of 6-phosphogluconate in the adrenal gland after injection of 6-AN at 50 mg kg^{-1}. After 3 h, 6-phosphogluconate levels started to increase and reached a near maximum of 3.52 mmol kg^{-1} after 6 h (Fig. 2). This increased value should be compared with the endogenous level of 16.5 μmol kg^{-1} in control glands (210-fold increase).

Effect of 6-AN on noradrenaline in hypothalamus and atrial tissue. The drastic effects of 6-AN on adrenal catecholamines seem to be specific to this tissue. No significant effects on noradrenaline stores were observed in hypothalamus or heart tissue (Table I).

Effect of 6-AN on adrenal catecholamines after drum stress. Immediately after a 14 h period of drum stress, the noradrenaline and adrenaline contents of the adrenal glands fell to 66 and 31 % of the respective control values. In the animals which received only a saline injection after cessation of the drum stress, resynthesis of adrenal catecholamines proceeded during the first 24 h at a rate of 49.2 nmol g^{-1} h^{-1}. The half life of catecholamine restoration in the adrenal gland was 20.4 h (calculated from the rate constant of total amine resynthesis).

TABLE I. Effect of 6-AN on noradrenaline stores in the hypothalamus and atria.

Time after injection of 6-AN (hours)	Noradrenaline, % of control	
	Hypothalamus	Atria
	74.0±12.9	102.6±11.0
4	130.5±26.7	84.8± 9.1
6	119.0± 7.6	— —
10	79.5±4.5	79.9± 4.6

6-AN was injected i.p. at 50 mg kg^{-1}. Values are expressed as percentages of those for controls injected with saline and killed after the same time intervals. Means ±S.E. for 3 rats. Pooled control values were 10.4±1.2 nmol g^{-1} and 10.8±1.1 nmol g^{-1} for hypothalamus and atria, respectively.

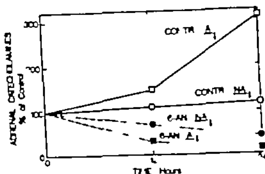


Fig. 3 Effect of 6-AN on adrenal catecholamines after drum stress. After cessation of the drum stress, the animals were divided into 2 groups. One group received 6-AN i.p. at 35 mg kg^{-1} while the other served as saline controls. Values are expressed as percentages of the catecholamine concentration after the cessation of the drum stress (adrenaline, 0.414 and noradrenaline, 0.347 µmol g^{-1}). Each point represents the mean for 3–9 animals. A adrenaline, NA noradrenaline.

this corresponds to a turnover time of 5.1 days, which is of the same order of magnitude as was obtained previously (Eränkö and Härkönen 1961). The rate of adrenaline recovery was 6.7 times as rapid as that of noradrenaline. These events are illustrated on a normalized scale in Fig. 3.

When administered immediately after the drum stress ceased, 6-AN effectively prevented restoration of noradrenaline and adrenaline stores in the adrenal glands. At 12 h after the injection of 6-AN at 35 mg kg^{-1} the adrenaline content was only 32 % of the value found immediately after the drum stress, and after 24 h the adrenals were almost depleted of adrenaline (Fig. 3). Likewise, noradrenaline stores fell on treatment with 6-AN, the fall being continuous instead of transient as it was in unstressed animals. Twenty-four hours after the injection, the noradrenaline concentration in the adrenal gland was reduced to 0.138 µmol g^{-1} as compared with 0.347 µmol g^{-1} immediately after drum stress. During this period, the noradrenaline stores in the saline-injected animals had increased to 0.399 µmol g^{-1}.

Effect of 6-AN on noradrenaline in hypothalamus and cervical ganglion after drum stress

The noradrenaline content in the superior cervical ganglion was not affected by the drum stress while in the hypothalamus, noradrenaline stores showed an insignificant increase to 138% ($p < 0.2$) of the control value (Table II).

After drum stress, 6-AN was without significant effect on noradrenaline stores in the superior cervical ganglion (Table III). In the hypothalamus, a 30 % decrease in the noradrenaline content was observed 24 h after the 6-AN injection (35 mg kg^{-1}), but the difference was not statistically significant ($p > 0.3$).

TABLE II Effect of drum stress on the noradrenaline concentration in the superior cervical ganglion and hypothalamus

	Noradrenaline concentration	
	Cervical ganglion (nmol per ganglion)	Hypothalamus (nmol g^{-1})
Control	0.50 ± 0.04	8.70 ± 0.80
Drum stress	0.50 ± 0.07	12.00 ± 2.05

Drum stress was carried out as explained in Methods. Means ± S.E. for 9 (controls) or 6 (drum stress) animals.

TABLE III Effect of 6-AN on the noradrenaline concentration in the hypothalamus and cervical ganglion after drum stress.

Time after drum stress and 6-AN injection (hours)	Noradrenaline content			
	Cervical ganglion (nmol per ganglion)		Hypothalamus (nmol g⁻¹)	
	Control	6-AN	Control	6-AN
12	0.53 ± 0.10	0.62 ± 0.10	10.7 ± 2.0	10.0 ± 1.7
24	0.63 ± 0.03	0.63 ± 0.06	$11.4 \pm .7$	$8.0 \pm 1.$

Immediately after the cessation of the drum stress, 6-AN was injected i.p. at 35 mg kg^{-1}. Means ±S.E. for 4–6 animals.

Effect of 6-AN on adrenal adrenaline content in vitro 6-AN did not affect adrenaline stores in the gland *in vitro* This is shown in Fig. 4 where the efflux of ^{3}H from slices preloaded with ^{3}H-adrenaline took place at the same rate whether the slices were treated with 6-AN or not

Effect of 6-AN on the uptake of noradrenaline by synaptosomes When synaptosomes were treated with 10^{-4} mol l^{-1} 6-AN at room temperature 6-phosphogluconate began to accumulate immediately At 1 and 2 h after the addition of 6-AN the level of 6-phosphogluconate in synaptosomes was increased by 4 and 6 times respectively (Fig. 5) Despite this pronounced rise, 6-AN did not affect the uptake of ^{3}H-noradrenaline by the synaptosomes (Fig. 6).

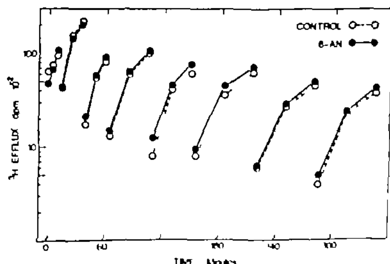
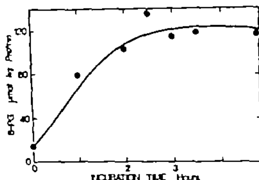


Fig. 4 Effect of 6-AN on the efflux of ^{3}H from adrenal slices after preloading with ^{3}H adrenaline Adrenal slices were incubated with ^{3}H adrenaline as explained in Methods After incubation the slices were divided into 2 groups to one 6-AN was added at 10^{-4} mol l^{-1} while the other served as control Incubation was continued at room temperature for up to 5 75 h The incubation volume was 1 0 ml and at various times after the addition of 6-AN, 200 µl samples of the incubation medium were removed for the determination of ^{3}H efflux. Extracellular accumulation and subsequently accelerated reuptake of ^{3}H-adrenaline were avoided by aspirating the incubation medium at predetermined intervals and replacing it with fresh medium. In the figure, this is indicated by the regularly spaced intervals between each 3-sample portion of the efflux curve. Results of 1 expt



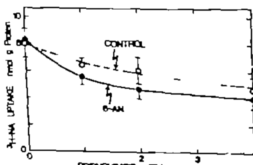
5. Accumulation of 6-phosphogluconate (6-PG) in synaptosomes after treatment with 6-AN in vitro. Synaptosomes were incubated at room temperature with 6-AN at 10^{-4} M. Each point represents the result of one determination.

ther did 6-AN affect synaptosomal ^{3}H-noradrenaline uptake when administered *in vivo*. When rats were injected with 6-AN at 50 mg kg^{-1} and killed 3 h later the synaptosomes isolated took up 10.93 ± 0.44 nmol noradrenaline g^{-1} protein min as compared with the control value of 11.39 ± 0.53 nmol g^{-1} protein min (means ± S.E. of 4 expts.).

Discussion

The results confirm the observation by Schacht *et al.* (1966) that 6-AN administered during fasting conditions depletes adrenaline stores in the adrenal gland *in vivo* whereas noradrenaline is only transiently lowered. These effects are seen only in the adrenals and may be due to a specific drug effect or could be secondary to the toxic effects of the drug. 6-AN causes hypothermia (see Lason 1970), a condition which increases the adrenal catecholamine turnover rate but does not cause any actual depletion of the amines (Leduc 1961. Gordon *et al.* 1966). The toxic effects of 6-AN include extrapyramidal-motor disorders which, by constituting a stress situation, might hypothetically lower adrenal amine stores. However motor symptoms were not manifested until about 4 h after the 6-AN injection, while the amine levels fell by 50 in 3 h. Although an effect on adrenal catecholamine stores due to a 6-AN-induced stress situation cannot be excluded, specific drug effects appear more probable.

The decrease in adrenal catecholamines induced by 6-AN *in vivo* appears to result from synaptic effects. The initial 50 reduction is most likely explained by an effect of 6-AN either on the adrenal nerves or directly on the medullary cells, since at this time the pentose



6. Effect of 6-AN on the uptake of ^{3}H-noradrenaline by synaptosomes in vitro. Synaptosomes were incubated with 6-AN at 10^{-4} M at room temperature. At various times after the addition of 6-AN, the ability of the synaptosomes to take up ^{3}H noradrenaline from the medium was tested as described in Methods. Each point is the mean ± S.E. of 4–10 expts.

phosphate pathway was still operative. The mechanism underlying this initial 6-AN effect remains unclear. The drug is hardly likely to affect amine uptake or storage or release mechanisms, as these parameters remained unaffected in adrenal slices and synaptosomes treated with 6-AN. However, the initial amine depletion observed after 6-AN appears to be specific for the adrenal glands, may be reversible and is known to occur with a simultaneous loss of ATP (Schacht *et al.* 1966). In this respect it differs from the subsequent phase of depletion which is characterized by a low amine : ATP ratio (Schacht *et al.* 1966).

The later sustained effect of 6-AN on adrenal amine stores occurred at the time when blockade of the pentose phosphate pathway was established, and exhibited an apparent selectivity for adrenaline. The reason for this is unknown but during resting conditions the increased amine synthesis due to reduced end-product inhibition (Nagatsu *et al.* 1964, Udenfriend *et al.* 1965, Stjärne 1966) allowed complete restoration of noradrenaline stores.

Even when the pentose phosphate pathway was blocked by 6-AN, amine uptake and release processes remained unaffected in synaptosomes and adrenal slices. These negative *in vitro* results would be understandable if the effects induced by 6-AN depend on intact innervation. Present results indicate that this may be the case: during resting conditions, 6-AN only transiently lowered noradrenaline stores in the adrenal gland, while sustained depletion took place when the amine turnover rate was increased by a preceding period of drum stress. Tetrahydropteridines act as cofactors of tyrosine hydroxylase (Nagatsu *et al.* 1964) and are maintained in a reduced state in a reaction involving NADPH. As the NADPH in neural tissue is largely formed via the pentose phosphate pathway (Härkönen and Kauffman 1974), and as tyrosine hydroxylase is the rate limiting step in catecholamine biosynthesis (Levitt *et al.* 1965), blockade of the pathway could result in retarded amine synthesis and loss of tissue catecholamines when the turnover rate is increased during a stress situation.

The supposed inhibition of tyrosine hydroxylase after blockade of the pentose phosphate pathway resulted in amine loss only in the adrenals and not in the superior cervical ganglion or in the hypothalamus. This might reflect differences in ability to reaccumulate secreted catecholamines: with the exception of the adrenal medulla, in which reuptake is low (see Stjärne 1972), neural tissue has a good ability for reuptake of amines (Haefely, Hürlimann and Thoenen 1965). However, blockade of the pentose phosphate pathway would be expected to produce pronounced effects especially in the adrenals since in this tissue, metabolism via the pathway is exceptionally active. If it is assumed that the linear increase in 6-phosphogluconate concentration 3 h after injection of 6-AN was due to complete inhibition of 6-phosphogluconate dehydrogenase, and that the oxidation of glucose-6-phosphate was the only source of 6-phosphogluconate, the rate at which glucose would enter the pentose phosphate pathway would be 0.91 mmol kg^{-1} wet weight h^{-1}. This value corresponds to appr. 37% of the normal hexose use in adrenal medullary slices (2.44 mmol kg^{-1} wet weight h^{-1}), which is considerably higher than estimates of the pentose phosphate pathway activity in brain (3–5%, Hostetler *et al.* 1970) and in superior cervical ganglion (16%, Härkönen and Kauffman 1974).

We conclude that NADPH generated in the pentose phosphate pathway may be important for the maintenance of the tyrosine hydroxylase activity needed for adequate biosynthesis

catecholamines during conditions of increased amine utilization. A closer study of the ect of 6-AN on amine turnover rates is indicated.

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References

ton, A. H. and D. F. Sayre, Possible sources of error in solvent extraction procedures for catechol-amines. *Europ. J. Pharmacol.* 1968, *4* 435–440.
ay G. A. A simple efficient liquid scintillator for counting aqueous solutions in liquid scintillation counter. *Analyt. Biochem.* 1960 *1* 279–285.
sob, O. and M. Härkönen, Distribution and concentration of adrenaline and noradrenaline in the adrenal medulla of the rat following depletion induced by muscular work. *Acta physiol. scand.* 1961 *51* 247–257
owinski, J. and L. L. Iversen, Regional studies of catecholamines in the rat brain I. The disposition of (³H) norepinephrine, (³H) dopamine and (³H) DOPA in various regions of the brain. *J. Neurochem.* 1966. *13* 655–669
ordon, R. S. Spector, A. Sjoerdsma and S. Udenfriend, Increased synthesis of norepinephrine and epinephrine in the intact rat during exercise and exposure to cold. *J. Pharmacol. exp. Ther.* 1966. *153* 440–447
aefely W. A. Hürlimann and H. Thoenen, Relation between the rates of stimulation and quantity of noradrenaline liberated from sympathetic nerve endings in the isolated perfused spleen of the cat. *J. Physiol. (Lond.)* 1965 *181* 48–58.
Härkönen, M. H. A. and F. C. Kauffman, Metabolic alterations in axotomized superior cervical ganglion of the rat. II. The pentose phosphate pathway. *Brain Res.* 1974. *65* 141 157
Härkönen, M. H. A. J. V. Passonneau and O. H. Lowry. Relationships between energy reserves and function in rat superior cervical ganglion. *J. Neurochem.* 1969 *16*. 1439–1450
Herken, H. K. Lange and H. Kolbe, Brain disorders induced by pharmacological blockade of the pentose phosphate pathway. *Biochem. Biophys. Res. Commun.* 1969 *36*. 93–100
Hostetler, K. Y. B. R. Landau R. J. White, M. S. Albin and D. Yashon, Contribution of the pentose cycle to the metabolism of glucose in the isolated perfused brain of the monkey. *J. Neurochem.* 1970 *17* 33–39
Kauffman, F. C. J. C. Brown, J. V. Passonneau and O. H. Lowry. Effects of changes in brain metabolism on levels of pentose phosphate intermediates. *J. biol. Chem.* 1969 *244* 3647 3653
Leduc, J. Catecholamine production and release in exposure and acclimation to cold. *Acta physiol. scand.* Suppl 183 1961
Levitt, M., S. Spector, A. Sjoerdsma and S. Udenfriend, Elucidation of the rate limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. *J. Pharmacol. exp. Ther.* 1965. *148* 1–8.
Loos, H. Untersuchungen zur Differenzierung der durch 6-Aminonicotinamid, einem Antimetaboliten des Nicotinamids, hervorgerufenen zentralnervösen Funktionsstörungen. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* 1970 *267* 135–169
Lowry O. H. N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* 1951 *193*. 265–275
Nagatsu, T. M. Levitt and S. Udenfriend, Tyrosine hydroxylase: the initial step in norepinephrine biosynthesis. *J. biol. Chem.* 1964 *239* 2910–2917
Schacht U. G. Schultz and G. Senft. Catecholamin- und ATP-Gehalt der Nebenniere nach Gabe von 6-Aminonicotinsäureamid. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* 1966. *253*. 355–363.
Stjärne, L., Studies of noradrenaline biosynthesis in nerve tissue. *Acta physiol. scand.* 1966. *67* 441–454
Stjärne, L., The synthesis, uptake and storage of catecholamines in the adrenal medulla. The effect of drugs. In *Catecholamines* Eds. H. Blaschko and E. Muscholl. Springer Verlag, Berlin, Heidelberg, New York 1972 pp. 231–269
Udenfriend, S., P. Zaltzman-Nirenberg and T. Nagatsu, Inhibitors of purified beef adrenal tyrosine hydroxylase. *Biochem. Pharmacol.* 1965 *14* 837–845
Weil-Malherbe, H. The estimation of total (free conjugated) catecholamines and some catecholamine metabolites in human urine. In *Methods of Biochemical Analysis* Ed. D. Glick. Interscience Publishers, John Wiley & Sons, New York, London, Sydney Toronto. 1968, pp. 293–326.

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We conclude that NADPH generated in the pentose phosphate pathway may be important for the maintenance of the tyrosine hydroxylase activity needed for adequate biosynthesis

ynert 1971 Daniels *et al.* 1974). Furthermore, the very low concentrations of Ca and ions in the adrenal vesicles (Borowitz *et al* 1965), as well as infrared (Pal and Maynert and electromagnetic (Daniels *et al.* 1974) spectra of medullary granule material argue a role for metal ions in an ATP–CA storage complex.

, assuming an ATP:CA ratio of 1 4 in the ATP–CA storage complex we ha e still ain the storage of the remaining 20–30 of the CA in the adrenal medulla which be ionically linked to the phosphate groups of ATP

basophil granules of mast cells have a high amine binding capacity both *in vitro* and (Uvnäs *et al.* 1970, Uvnäs and Bergendorff 1973). From a storage point of view these s contain negligible amounts of ATP Their matrix consists of a heparin–protein x with the properties of a weak cation exchanger material, capable of binding both ic cations and biogenic amines. Experimental evidence indicates that endogenous ine is stored in ionic linkage to free carboxyls in the protein part of this granule complex and is released by cation exchange when these ionic sites become exposed cellular cations (mainly sodium) during the exocytosis of the mast cell granules (for ws see Uvnäs 1975).

e present study the ability of bovine adrenal medulla granule material which had pleted of CA and ATP by dialysis to bind inorganic and organic cations has been ated The material has the properties of a weak cation exchanger and the binding y suffices to explain the storage of 20–30 % of the normal CA content of the bovine ary granules.

Methods

... of granule material from adrenal medulla

adrenal medullary granules were isolated according t Poisner and Trifaró (1967). The adrenals ught from the slaughter house on ice and immediately on arrival (about 3 h after death) the medul- removed and homogenized in ice-cold 0.32 M sucrose using glass homogenizer After centrifuga- 10 min at 800 g the supernatant as removed and filtered under ~100 p nitrogen pressure series of 25 mm nucleopore filters placed in Sartorius filter apparatus, type SM 16223. The pore d were 3 1 2, 0 6, 0 45 and 0 2 µm After passing the 0 2 µm filter the filtrate as centrifuged for t 20 000 g and the resulting sediment as suspended in 4 ml of ice-cold deionized water The nt was then dialyzed against 4 l of deionized water under continuous rocking for 24 h All the ns described above were performed in the cold room (4 + 4°C). The dialyzed material, found tically depleted of CA and ATP was diluted ith deionized water to 7 5–9.5 ml before use in the ptake studies This suspension ill be referred to below as the granule suspension.

...udies

...f Na at pH 7 4 Fresh 0 2 M sodium phosphate buffer as prepared by titrating 20 ml of 1 M ith concentrated H_3PO to ~pH 7 3, and diluted to 100 ml ith deionized water he pH was to 7 30 with conc H_3PO_4 From this 0 2 M buffer stock solution 9 ml samples were prepared ng 200, 60, 20, 6, 2, 0 6 and 0 2 µmol of Na /ml T each sample 25 µl of ^{22}NaCl (100 µCi/ml) was T 1 ml of each of the samples was added 1 ml of granule suspension and the pH as adjusted to itration ith 1 M NaOH. The NaOH added as taken into account at hen calculating the uptake na by the granule material

l ml samples of granule material suspended in sodium phosphat buffer were filtered through ne filter (UM 2, Diaflo, Amicon, 25 mm) in the Sartorius filter apparatus under nitrogen pressure The UM 2 filter was stated to retain materials with molecular weights bove 1 000. The filtrat H ~7 4 (at room temperature). The UM 2 filter ith its moist layer of retained granule material as d from the filter apparatus and folded manually to fit the opening of tared plastic tube (11 55 mm)

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The Ability of ATP Free Granule Material from Bovine Adrenal Medulla to Bind Inorganic Cations and Biogenic Amines

By

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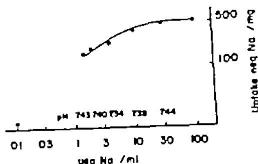
Abstract

UVNÄS, B and C. H ÅBORG *The ability of ATP free granule material from bovine adrenal medulla to bind inorganic cations and biogenic amines* Acta physiol. scand 1977 99 476–483

Bovine adrenal medullary granules isolated by millipore filtration were depleted of CA and ATP by dialysis. The resulting material showed an ability to bind inorganic cations and biogenic amines in a concentration-dependent manner The similarity of the uptake curves, the identical uptake maxima and the narrow pH range (between 4–7) over which the uptake of the inorganic and organic cations took place indicated binding of these ions to common sites. In addition, the fact that all the uptake curves fitted the Rothmund-Kornfeld equation for cation exchangers *corroborated the cation exchanger properties of the dialyzed* granule material. The CA binding capacity corresponded to 20–30 of the normal CA content of bovine *medullary granules*.

The concomitant occurrence in adrenal medullary granules of catecholamines (CA) and adenosine nucleotides, mainly adenosine-5 -triphosphate (ATP) has led to the widely accepted hypothesis that the CA are stored in the granules in some kind of a complex with this nucleotide. Since at pH 7 4 CA are positively charged and ATP carries 4 negative charges, it has been suggested that CA are ionically bound to the polyvalent nucleotide (Blaschko *et al* 1956, Falck *et al* 1956) However since the ratio—total positive CA charge/total negative nucleotide charge—was found to be 1 some other kind of storage mechanism had to be assumed for part of the CA According to Hillarp (1960), in the bovine adrenal medulla at least 20 % of the CA had thus to be stored in a manner not involving ATP

From comparative physico-chemical studies on aggregates formed *in vitro* between CA and ATP—with or without the admixture of alkaline earth metal ions (Ca and Mg)—and of adrenal medullary granules, Berneis *et al* (1971) concluded that aggregate formation with ATP may play an essential role in the uptake and storage of CA in adrenal medullary granules. Recent studies using high resolution proton and phosphorus electromagnetic spectroscopy were considered to confirm the occurrence in bovine medullary granules of an ATP–CA complex and to support the idea of an ATP–CA ratio of 1 4 in this complex (Pai



Concentration dependent uptake of ... dialyzed granule material from adrenal medulla suspended in sodium phosphate ... pH 7.4. Observed uptakes ●—● ... calculated for pH 7.4 ●—● from the Rothmund-Kornfeld equation; β 1 K= ... U_{max} 5?0 neq mg.

Results

... of inorganic cations and biogenic amines

... concentration dependence. As illustrated for sodium (Na) in Fig. 1 and for noradrenaline ...) in Fig. 2, all the inorganic cations (Na Ca++) and biogenic amines (PhEA, TA, NA, ...) studied were taken up from the suspension medium by the granule material. The ... uptake was observed to be concentration-dependent within a cation concentration range ... between 0.01 and 30 μeq/ml. The maximal uptake capacity varied somewhat between the ... batches of the granule material used, in most cases it was between 400 and 500 neq/mg dry weight.

pH-Dependence. The cation uptake was strongly influenced by the pH of the suspension medium. Lowering the pH from 7–8 to 4–5 abolished the storage capacity of the granule material, as illustrated for Na and NA in Fig. 3

... for free acid groups

Titration of granule suspensions with 0.1 M HCl showed a consumption of between 400–... neq of acid/mg dried material between pHs 7.4 and 5.0. The titration values for each batch of granule material agreed well with its cation uptake capacity

Application of the Rothmund-Kornfeld equation

The Rothmund-Kornfeld equation (Eq. 1) is an empirical modification of the Law of the Mass Action valid for cation exchangers (Samuelsson 1952). The equation implies that, when two cations compete for the ionic sites of the exchanger plotting the log of the ratios

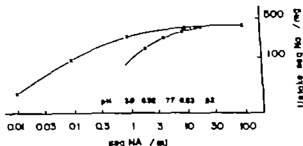


Fig. 2. Concentration dependent uptake of NA by dialyzed granule material from adrenal medulla suspended in NA solution at pH ~ 7. Observed uptakes (—). Uptake calculated (●—●) for pH 7.4 from the Rothmund-Kornfeld equation β 0.7 K 1.26 10⁻⁴ U_{max} ... neq mg

in order to minimize loss of the material when it was scraped down into the tube (with a stainless steel spatula). The previously weighed tube was reweighed with its moist content, then dried over P_2O_5 *in vacuo* to constant weight (70 h), and again weighed. One ml of 0.4 M HCl was added to the tube which was then closed with a plastic stopper. The stopper was secured with tape and the tube was heated to about 60°C whilst shaking vigorously. After centrifugation for 30 min at 3 000 g 0.5 ml of the supernatant was neutralized with 0.4 ml of 0.5 M NaOH and 15 ml of scintillation fluid was added (0.4% 2,5-diphenyl-oxazole, 0.01% 1 4-bis-2(4-methyl-5-phenyl oxazolyl)-benzene in a mixture of 50% toluene and 50% ethylene glycol monoethyl ether).

To determine the ^{22}NaCl radioactivity in the suspension fluid after its passage through the UM 2 membrane, 25 μl was taken from each sample of the filtrate. These reference samples were dried and treated exactly as described above for the granule material. The determination of the ^{22}NaCl activity in the filtrates, together with the (careful) weighing procedures enabled a reliable estimate of the uptake of sodium (and amines) by the granule material to be made. When calculating the uptake, corrections were made for changes in the concentration of sodium (or the amine) in the suspension fluid due to the uptake by the granule material.

Uptake of amine at pH 6–7

Solutions of the amines were titrated to pH 6–7 with 5–6% H_2SO_4 and diluted to the desired concentrations with deionized water in a manner analogous to that described above under sodium uptake. H_2SO_4 was used since it yields water soluble salts, prevents oxidation and has maximum buffering capacity at pH 7 (pK_a = 6.9). Considering these advantages, the drawback of a rather poor buffering capacity was knowingly accepted.

Influence of pH on the uptake of sodium

0.1 M sodium phosphate buffer was prepared by titrating 1 M NaOH with conc. H_3PO_4 to pH 7.8. Equal volumes of granule suspension and buffer solution were mixed and appropriate amounts of ^{22}NaCl were added. During stepwise acidification with conc. H_3PO_4 ml samples were taken at desired pHs for the determination of sodium uptake as described above. To retain constant Na^+ ion concentrations during the titration the H_3PO_4 solution contained 0.05 M sodium chloride and the same concentration of ^{22}NaCl as the sample to be titrated.

Influence of pH on the uptake of amines

Solutions of the amines were titrated to pH 7 with 5–6% H_2SO_4. Appropriate amounts of radioactive amine were added. During stepwise acidification with conc. H_3PO_4, samples were taken at desired pHs for the determination of amine uptake as described above.

Titration of granule material for free acid groups

2 ml of granule suspension—in deionized water or 0.1 M NaCl—was titrated at 0°C, and at room temperature to pH 9.1 with 0.1 M NaOH and then with 0.1 M HCl to pH 3.0.

To minimize possible disturbing effects of various enzymes and of bacterial growth, all procedures described above, except titrations were performed in the cold room (+4°C). An Agla micrometer syringe was used for all titrations.

Materials

^{22}Na (sodium chloride in aqueous solution carrier free, 100 mCi/mg Na), *DL (7-³H) adrenaline* (91 mCi/mg), *L-(7-³H) noradrenaline* (43.3 mCi/mg), *tyramine-(1-¹⁴C) hydrochloride* (314 μC/mg), *β-phenylethylamine-(1-¹⁴C)*, HCl (45 μCi/mg), *(ring-2-¹⁴C) histamine dihydrochloride* (50 mC/m mol) and ^{45}C (calcium chloride in aqueous solution, 10–40 mCi/mg Ca), were obtained from the Radiochemical Centre, Amersham, England.

Abbreviations

A – Adrenaline
NA – Noradrenaline
TA – Tyramine
PhEA – Phenylethylamine
DA – Dopamine
CA – Catecholamine
ATP – Adenosine-5'-triphosphate
Hi – Histamine

Poisner, A. M. and J. M. Trifaró, The role of ATP and ATPase in the release of catecholamines from the adrenal medulla. I. ATP-evoked release of catecholamines, ATP and protein from isolated chromaffin granules. *Molec. Pharmacol.* 1967. 3. 561–571.

Samuelson O., *Ion exchangers in analytical chemistry.* Almqvist & Wiksell, Uppsala 1952.

Uvnäs, B. The molecular basis for the storage and release of histamine in rat mast cell granules. Minireview. *Life Sci* 1974. 14. 2355–2366.

Uvnäs, B. and C.-H. Åborg, An in vitro-formed protamine-heparin complex as a model for a two-compartment store for biogenic amines. *Acta physiol. scand.* 1976. 96. 51[illegible].

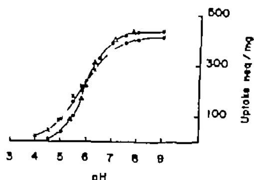


Fig. 3 Influence of pH on the uptake of Na and NA by dialyzed granule material from adrenal medulla. Conc. Na 100 μeq/ml, NA 70 μeq/ml. Observed uptakes for Na △—△ and NA — Uptakes calculated according to the Rothmund-Kornfeld equation from the conc. curves in Fig. 1 and for Na ○—○ and for NA+ ●—● respectively

between the concentrations of the two cations in the exchanger and the log of the corresponding concentrations of the cations in the suspension medium should give a straight line.

Assuming that in the present expts. the granule material behaves like a cation exchanger the two cations competing for the binding sites will be hydrogen ions and the cation under study. Since the maximal uptakes for these two ions in the same batch of granule material are the same, and the pH and the cation uptake are known the validity of the exchanger equation for the cation binding of the granule material can be tested.

As can be seen from Fig. 4 the uptake values obtained for all the inorganic and organic cations studied fit the cation exchange equation. It is noteworthy that the monoamines A NA TA and PhEA had practically identical affinities for the binding sites, in contrast to the considerable differences between the monovalent Na and the divalent Ca ions.

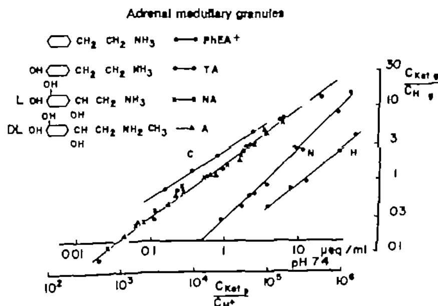


Fig. 4 Plotting of the ratios between inorganic and organic cation concentrations and hydrogen ion concentrations in dialyzed granule material against the corresponding cation concentration in the suspension medium. For symbol see the figure.

Note For graphic constructions of concentration and pH curves at pH 7.4 the cation conc. in suspension medium at pH 7.4 (line above the abscissa) is related to the ratio cation conc./hydrogen ion conc. in the suspension medium.

$$\frac{[B_r]}{[A_r]} \cdot \left(\frac{[A_w]}{[B_w]}\right)^{\beta} = K \qquad (1)$$

1 The Rothmund-Kornfeld equation, A_r and B_r = ions in ion exchange material (resin), A_w and B_w = solution, β and K empirical factors

[illegible] seems to have a lower affinity for the granule binding sites than do the other [illegible] studied.

From the concentration or pH curves the empirical factors K and β in the Rothmund-[Korn]feld equation can be calculated. The pH curves calculated from the experimentally [obta]ined concentration curves in Fig. 1 and 2 fit the experimentally obtained curves well [(Fig.] 3).

In our studies of the concentration-dependence of the uptake of the amines we were [not a]ble to keep the pH constant at neutrality. With diminishing amine concentrations there [wa]s a progressive slight acidification towards pH 6. Due to the strong pH dependence of [the] uptake, even a slight fall in pH resulted in a considerably reduced uptake. This fact is [illu]strated in Fig. 2. A recalculation for pH 7.4 according to the Rothmund-Kornfeld [equ]ation yields a concentration range for the uptake of NA considerably wider than experi[ment]ally obtained.

Discussion

[T]he present results demonstrate that granule material from bovine adrenal medulla prepared [b]y millipore filtration according to Posner and Trifaró (1967) retains an ability to bind [in]organic cations and biogenic amines even after dialysis against water. Dialysis depleted [th]e material of CA and ATP—as determined chemically—and presumably of other diffusible [in]organic and organic materials. In most of our expts. the dialyzed material was used for [up]take studies without further purification. Removal of undissolved material, and thereby [m]ost of the insoluble membrane fraction, by high speed centrifugation (100 000 g [...] min) reduced the uptake capacity of the remaining soluble material t[o] only a minor degree. The uptakes of both inorganic and organic cations were concentration-dependent within [a] concentration range of about 0.01–30 μeq/ml, at pH 7.4, and reached identical maximal [leve]ls in the same batch of material. The uptakes were strongly dependent on pH, with a [com]plete breakdown of the cation binding capacity on lowering the pH from 7–8 to 4–5. [T]he uptake capacity at 7–8 corresponded fairly well to the number of weakly acid groups in [th]e granule material, as indicated by the HCl consumption on titration of the granule material [fr]om pH 7 to pH 4. Taken together, the observations agree with the assumption that the [u]ptake of cations by the granule material depends on the presence of weak anionic groups, [pr]obably carboxyls. The demonstration that the uptake curves for all the cations studied [fi]t the Rothmund-Kornfeld equation corroborates the idea that the granule material behaves [li]ke a [w]eak cation exchanger. The identical affinities of the monoamines (PhEA, TA, NA, A) [s]trongly suggest that the binding forces mainly emanate from the ionized amino groups on [t]he side chain of the amines, the hydroxyl groups in the aromatic ring or in the side chain [pl]aying only an insignificant role.

The localization of the ionic binding sites in the granule material is unclear After the extensive dialysis and subsequent high speed centrifugation to which the granule material was subjected mainly chromogranins remain. Since chromogranins are unusually acid proteins, they will contain considerable numbers of free carboxyl groups capable of binding cations. However to what extent anionic binding sites may also originate from solubilized granule membrane constituents, mucopolysaccharides, glycoproteins etc. remains to be studied.

It is notable that the observed cation uptake capacity corresponds to about 20–30 of the normal CA content of the bovine adrenal medullary granules, *i.e* about the same percentage of the CA store which according to Hillarp (1960) could not be bound to granule ATP However until the localization of the cation binding sites is established the biological implications of the present observations are obscure. It might be appropriate to recall our previous observations that the matrix of the basophil mast cell granules has the properties of a weak cation exchanger and that the storage and release of histamine in mast cells can be explained as cation exchange processes. Whether or not similar cation exchange mechanisms play a role in the storage and release of the catecholamines in the adrenal medulla remains to be seen. Similarly the physiological importance of the observed cation exchanger properties of material obtained from neuronal adrenergic vesicle preparations (to be published) is not yet clear

As will be described in a later publication, the medullary granule material behaves *in vitro* like a two-compartment store for biogenic amines. With higher concentrations of aromatic amines than those described in the present paper (50–300 μeq/ml) increasing amounts (proportions) of the amines were taken up Also in this respect the medullary granule material shows similar biogenic amine storing properties to those shown for a protamine heparin complex (PHC) by Uvnäs and Åborg (1976) and for mast cell granules by Uvnäs and Åborg (to be published) The functional implications of these observations will be dealt with in a coming publication.

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References

BERNEIS, K. H., M DA PRADA and A. PLETSCHER, A possible mecha ism for upta of biogenic mines by storage organelles. Incorporation nto nucleotide–metal aggregates *Experientia* (Basel) 1971 27 917 918

BLASCHKO, H G V R. BORN, A D'IORIO and N R EADE, Observations o the dist bution of catecholamines and adenosine-triphosphate in the bovine adrenal medulla *J Ph siol* (Lond) 1956 *133* 548 557

BOROWITZ, J L., K. FUWA and N WEINER, Distributio of metals and catecholamines bov ne adrenal medulla sub-cellular fractions. *Nature* (Lond.) 1965 205 4–41.

DANIELS, A., A. KORDA, P TANSWELL, A. WILLIAMS and R J P WILLIAMS, The internal structure of the chromaffin granule. *Proc roy Soc B* 1974 *187* 353–361

FALCK, B., N Å. HILLARP and B. HÖGBERG, Content of tracellula dist bution of adenosine triphosphate in cow adrenal medulla. *Acta physiol. scand* 1956 *36* 360–376.

HILLARP N Å., Different pools of catecholamines tored the adrenal medulla. *Acta ph siol. scand* 1960. *50* 8–22.

PAI, V S. and E. W MAYNERT Interactions of catecholamines w th adenosine-t phosphate i solutions and adrenal medullary granules. *Molec Pharmacol.* 1971 8 8 –87

POISNER, A. M. and J. M. TRIFARÓ, The role of ATP and ATPase in the release of catecholamines from the adrenal medulla. I. ATP-evoked release of catecholamines, ATP and protein from isolated chromaffin granules. *Molec. Pharmacol.* 1967. 3. 561–571.

SAMUELSON, O. *Ion exchangers in analytical chemistry.* Almqvist & Wiksell, Uppsala 1952.

UVNÄS, B. The molecular basis for the storage and release of histamine in rat mast cell granules. *Mini-review Life Sci.* 1974. 14. 2355–2366.

UVNÄS, B. and C.-H. ÅBORG, An in vitro-formed protamine-heparin complex as a model for a two-compartment store for biogenic amines. *Acta physiol. scand.* 1976. 96. 512–525.

Acta physiol scand. 1977 99 484–495
From the Department of Physiology University of Göteborg, Sweden

Contractility, Muscle Mass and Agonist Sensitivity of Isolated Portal Veins from Normo- and Hypertensive Rats

By

MORLEY C. SUTTER and BENGT LJUNG

Received 6 October 1976

Abstract

SUTTER M C. and B LJUNG *Contractility muscle mass and agonist sensitivity of isolat portal veins from normo- and hypertensive rats* Acta physiol scand. 1977 99 484–49

Properties of the longitudinal smooth muscle of portal veins from normotensive Wistar rats, adult (NCI nd young (NCR_y) spontaneously hypertensive Okamoto rats, adult (SHR) and young (SHR_y) and adu Wistar rats with renal hypertension (RHR) were studied vitro and histologically Some aortic strips fro SHR and SHR_y were compared with controls. In response to noradrenal ne (NA) and acetylcholine (ACl greater maximum force was developed by veins from all hypertensive groups than by those from contr rats. Cross-sectional area of the longitudinal muscle of veins from SHR but not SHR_y nor RHR greater than control. Maximum stress n response to agonists was greater in both SHR nd RHR tha NCR. ED_{50}-values for NA and ACh were lower in port l veins from SHR tha NCR but not from RHI nor SHR compared to co trols. Denervation did not bolish any of the differences between SHR an NCR. Aortic strips from SHR developed less maximum force t NA and ED_{50} was greater than those fro NCR *i.e.* opposite t the f ndings n portal veins. Low levels of external Ca reveal ltered calcium handlin in veins from SHR compared t controls.

It is concluded that portal veins from hypertensive rat are functionally different from those of nor motensive rats and d ffer n SHR compared to RHR It is suggested that the altered f nctional propertie of portal vein, but not of aorta in several respect resemble those of rterial resistance vessels The im plications of these f ndings are discussed in terms of mechanisms f hypertension in these mal models.

The spontaneously hypertensive rat (SHR) of the Okamoto strain is a much-studied model of essential hypertension. The pathogenic mechanism of this type of hypertension has been suggested to be hypertrophy of the vascular smooth muscle which is triggered by inter mittent rises in arterial pressure which can be ascribed to sympathetic hyperreactivity (Folkow *et al* 1973 Hallbäck 1975). Along with the development of the structural changes the hyper tension is becoming increasingly sustained and also accentuated by a positive feed-back relationship between functionally induced pressure rises and the hypertrophy of the pre-capillary resistance vessels. Finch and Haeusler (1974) however have presented evidence for the existence of altered excitation–contraction coupling in the perfused mesenteric artery

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-paration of these animals. On the other hand, Mulvany and Halpern (1976) have shown no correlation between the increased maximum force and the vascular wall thickness in isolated small mesenteric arteries of SHR as compared to normotensive Wistar Kyoto rats. The question of the etiology of the vascular alterations in hypertension is not settled, in fact both hypertrophy and enhanced contractility may be involved.

There have been many studies of isolated blood vessels from hypertensive animals. Usually large arteries are used and the question arises as to how closely they functionally resemble resistance vessels. Furthermore, it is always uncertain whether any differences observed in arteries from hypertensive animals are a consequence or a cause of the high arterial pressure.

Recently analyses of pressor responses to noradrenaline (NA) of consecutive segments of the vascular bed of isolated rat hindquarters perfused with solutions containing low or normal amounts of calcium have indicated that contraction of the peripheral resistance vessels is much more dependent on extra-cellular calcium than is contraction in more proximal vessels (Folkow *et al.* 1976). The mesenteric portal vein is also highly dependent on extra-cellular calcium for both its myogenic spontaneous activity and agonist-induced contractions (for ref. see Collins, Sutter and Teiser 1972). Contractions of aortic strips in response to various agonists are much less dependent on external calcium (Hudgins and Weiss 1968, Devine, Somlyo and Somlyo 1972). In this and other regards, the peripheral resistance vessels seem much more analogous to the mesenteric-portal vein *in vitro* than they are to aortic strips (*cf.* Rhodes and Sutter 1971, Folkow *et al.* 1977, Ljung 1977).

The present expts. were designed to study the rat portal vein, a vessel which is not exposed directly to the elevated systemic arterial pressure, in normo- and hypertensive rats and in a few cases to compare it to aortic strips from the same animals. The results indicate that portal veins from hypertensive rats differ from those of normotensive rats and that portal veins differ in one type of hypertension to another.

Methods

Animals

Adult male spontaneously hypertensive rats of the Okamoto strain (SHR) were approximately 6 months of age, weighed from 250–300 g and had mean arterial blood pressures (B.P. measured in the tail artery after recovery from light ether anesthesia) of at least 135 mmHg. The young male SHR were 6–8 weeks of age, weighed 135–155 g and had mean arterial B.P. of 105–115 mmHg. Normotensive control rats (NCR) were male Wistar rats matched by weight. The adult NCR's had mean B.P. of 105–120 mmHg and the young NCR (NCR$_y$) had mean B.P. of 95–105 mmHg. Renal hypertensive rats (RHR) were adult male Wistar rats made hypertensive by partial occlusion of the left renal artery by a silver clip (Lundgren 1974). They were used 2 months after clipping and had B.P. of 150–210 mmHg. Control animals were weight matched male Wistar rats (NCR). All animals were delivered by Møllegaard Hansens Avlslaboratorier Denmark. They were fed a standard diet (Anticimex) and given drinking water *ad lib.*

Organ-bath experiments

The rats were killed by a blow on the head. The portal vein was dissected free of adherent tissue, incised along its length to form a strip, and tied at both ends with silk. The vein was then excised, mounted in an organ bath and attached to a Grass FT 03 force-displacement transducer under 5 mN passive force. One control vein and one vein from a hypertensive animal were always mounted in the same organ bath. Force developed by the vein was recorded on a Grass Model 7 polygraph and the force signal was electronically integrated over 1 min periods on a separate channel.

In a few expts. helical strips were prepared from the thoracic aorta and mounted under 10 mN passive force for recording of responses as with the portal vein.

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Abstract

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Properties of the longitud nal smooth muscle of portal veins from normotensive Wistar rats, d lt (N and young (NCR_y) spontaneously hypertensive Okamoto rats, adult (SHR) and young (SHR) nd a Wistar rats with renal hypertension (RHR) were studied in vitro nd histologically Some aortic t ps fr SHR and SHR_y were compared with controls In response to noradrenal ne (NA) nd acetylchol ne (A greater maximum force was developed by veins from all hypertensive groups tha by those from con rats. Cross-sectional area of the lo gitudinal muscle of veins from SHR but not SHR_y nor RHR greater than control. Maximum stress in response t gonist was greater in both SHR and RHR t NCR. ED_{50}-values for NA and ACh were lower in portal veins from SHR than NCR but not from R nor SHR_y compared to controls. Denervation did not abolish any of the d fferences bet een SHR NCR Aortic strips from SHR developed less maximum force to NA nd ED_{50} was greater than those fr NCR, *i e* opposite to the findings i portal veins. Low levels of external Ca reveal altered calcium handl in veins from SHR compared to controls

It is concluded that portal veins from hypertensiv rat re functionally different from those of n motensive rats and differ in SHR compared t RHR. It is suggested that the altered functional propert of portal ein, but not of aorta, in several respects resemble those of arterial resistance essels The plications of these f ndi gs are discussed in terms of mechanisms of hypertensio these animal mode

The spontaneously hypertensive rat (SHR) of the Okamoto strain is a much-studied mod of essential hypertension The pathogenic mechanism of this type of hypertension has bee suggested to be hypertrophy of the vascular smooth muscle, which is triggered by inte mittent rises in arterial pressure which can be ascribed to sympathetic hyperreacti ity (Folko *et al* 1973 Hallbäck 1975) Along with the development of the structural changes the hype tension is becoming increasingly sustained and also accentuated by a positive feed-bac relationship between functionally induced pressure rises and the hypertrophy of the pre capillary resistance vessels. Finch and Haeusler (1974), however have presented evidenc for the existence of altered excitation–contraction coupling in the perfused mesenteric arter

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[T]ABLE I. Maximum peak force, and mean force levels obtained by integration, during spontaneous activity of the portal vein from normotensive and hypertensive rats and maximum force developed in response to noradrenaline or acetylcholine as determined from complete concentration-response curves. Mean values ±S.E. Number of observations (n) given within brackets. Maximum responses were not always sought in each tissue hence the sum of ( ) of the last two columns does not always equal the ( ) of the first column. For definitions of groups see Methods. * indicates p < 0.05

[Gr]oup	Spontaneous activity		Maximum response to agonist (mN)	
	Maximum force (mN)	Mean force (mN)	Noradrenaline	Acetylcholine
[N]CR	*5.8±0.4 (20)	*3.4±0.3 (19)	12.0±1.0 (11)	15.8±1.4 (6)
[S]HR	*9.8±0.5 (14)	*5.7±0.4 (11)	22.3±0.5 (5)	21.2±0.9 (6)
[R]HR	8.0±0.5 (6)	5.1±0.6 (6)	16.8±0.5 (6)	23.9±1.1 (3)
[N]CR$_y$	6.0±0.6 (9)	4.3±0.7 (9)	*13.2±0.8 (7)	
[S]HR$_y$	6.2±0.6 (9)	3.9±0.5 (9)	*15.7±0.7 (7)	
[N]CR$_{sym}$	5.6±1.7 (5)	3.3±1.1 (5)	9.7±3.7 (5)	
[S]HR$_{sym}$	10.5±1.0 (9)	6.4±0.9 (9)	20.6±1.8 (9)	
[S]HR$_{sham}$	6.8±0.8 (4)	5.0±1.1 (4)	21.0±0.9 (4)	
[S]HR$_{sham+pro}$	6.0±0.8 (4)	4.6±0.5 (4)	16.5±2.0 (4)	
[S]HR$_{sham+pro}$	6.5±1.0 (4)	4.4±0.7 (4)	20.4±1.2 (4)	

...t concentration and with no agonist added. Between contractions complete relaxation ...the baseline of passive force occurred. Low concentrations of NA and ACh induced ...citatory responses which were characterized by increased rate and amplitude of the phasic ...tivity. With increasing concentration of agonists the phasic contractions tended to fuse ...d at maximum responses a smooth, maintained level of active force was regularly recorded. The peak spontaneous force expressed as a percentage of the maximum force induced ...agonists was comparable in the different groups of rats and was found to range between ...and 50 per cent.

Table I summarizes the results obtained when spontaneous contraction and maximum ...ponses to agonists were quantitated in absolute terms. The spontaneous activity was ...ater in SHR and RHR veins compared to NCR veins both with regard to peak force of ...individual contractions and to mean force obtained by integration. Similarly the maximum ...k force developed in response to NA or ACh was greater in SHR compared to NCR ...ns. Sympathectomy produced no reduction of force developed during spontaneous con...ctions of SHR veins nor in their response to NA. Protriptyline seemed to reduce the peak ...rce of individual spontaneous contractions but did not significantly alter mean force ...loped spontaneously in veins from SHR nor to affect maximum force developed in ...onse to NA. As i[n] young rats, veins from SHR$_y$ and NCR$_y$ were similar in terms of ...e developed during spontaneous contractions but the maximum force in response to NA ...larger in SHR$_y$ veins.

...n addition to the studies on isolated portal vein preparations the maximum force de...ped in thoracic aortic strips in response to NA was compared for 5 NCR and 5 SHR rats. ...strips from NCR contracted more strongly than those from SHR, 15.3±2.1 and 12.6± ...mN (mean ±S.E.), respectively (p = 0.002 by paired t-test), and this is opposite to the ...ings in the portal vein. Maximum contractions produced by NA were also greater in

The fluid bathing the tissues was of the following composition in mM NaCl 122, KCl 4.73, $NaHCO_3$ 15.5 KH_2PO_4 1.19 $MgCl_2$ 1.19 glucose 11.5 $CaNa_2$-versenate 0.026. A $CaCl_2$ concentration of 2.5 mM was designated "normal". The solution was continuously bubbled with 4% CO_2 in O_2 and kept at 38 C.

The tissues were allowed to stabilize for at least 1 h before responses were induced. Agonists were added in volumes of 1 per cent of the bath fluid. Cumulative concentration–effect curves were made by proceeding from lowest to highest concentration of agonist and allowing each concentration to be in contact with the tissue for min. The average integrated force developed during the min exposure period minus the mean spontaneous force developed during the preceding 2 min control period was then used as the effect of the drug. These values were entered into a computer program which calculated the log ED_{50} value for each concentration–effect curve by a least-squares fit to a true hyperbolic function.

In expts. where calcium concentration was altered the protocol was as follows, a concentration–effect curve was first obtained in normal 2.5 mM calcium concentration and then the bath solution was changed into one with a different calcium concentration. After 30 min in the altered Ca solution another NA concentration–effect curve was obtained. The sequence of calcium concentrations was 2.5 mM (normal), 0.0, 0.1, 0.2, 0.4, 0.8 mM of added calcium and then back to normal calcium.

The following agonists were used: noradrenaline hydrochloride, (l-arterenol, Sigma) and acetylcholine chloride (Merck). All concentrations are expressed in terms of the base. Noradrenaline (NA) solutions were made up daily from a stock solution kept refrigerated and acidified with 10 mM HCl. The stock solution of acetylcholine (ACh) was kept frozen until the day of its use when it was diluted appropriately. In all cases agonists were diluted in saline.

In expts. where ACh was the agonist, phentolamine, 0.3 μM was constantly present in the bath fluid to inhibit any adrenergic response due to ACh-induced release of catecholamines from the tissue. In a few expts. on denervated portal veins ($SHR_{den+ptp}$) and on veins from sham-operated animals ($SHR_{sham+ptp}$) protriptyline 0.1 μM was present in the bathing fluid.

Surgical procedure

The portal vein of 20 rats was denervated as described previously (Johansson et al. 1970) and the vein used 10–14 days later. Controls (SHR_{sham}) were sham-operated on the same day as experimental animals. The criteria for successful sympathectomy was a response to a 1 min transmural stimulation with square wave pulses (8–16 Hz, 15 V, 0.8 ms) which was 10% or less of the response in a non-denervated vein or of the maximum response to NA.

Histology

At the end of the organ bath expt. the bath was lowered and a beaker filled with Bouin's solution brought into position and the tissues were fixed overnight (16 h) while attached to the force transducers at the length which prevailed during the expt. The portal veins were then transferred to individual vials filled with Bouin's fluid and stored until processed further 0.5–3 weeks later. The fixed tissues were dehydrated in alcohol, cleared in xylene, embedded and sectioned (5 μm) perpendicularly to the longitudinal axis of the vessel. Sections corresponding to the mid-point along the length of the portal vein preparation were selected and stained with Weigert's hematoxylin and van Gieson methods.

The sections were photographed in a Zeiss photo microscope at 400× magnification. A series of coded prints and a simultaneously obtained photograph of a calibration scale were provided to the examiner who traced the cross-sectional area of the longitudinal muscle layer of the portal vein onto heavy duty transparent paper. Likewise a square corresponding to 1 mm² was prepared. The tracings were cut, weighed, the code was deciphered and the cross-sectional areas, expressed in mm² were calculated for the effector tissues.

Statistics

Results in control and experimental groups were compared using Student's t test for group data and occasionally where appropriate, for paired data. A p-value of less than 0.05 was accepted as showing significant difference.

Results

Contractile force

In all rat portal veins studied, spontaneous phasic contractions at a rate of approximately 2–6 contractions per min were recorded in the control situation, i.e. at normal (2.5 mM)

ervated veins from NCR due to elimination of any trophic influence exerted by the omotor nerves. Surgery on the portal ein led to an increased variability of cross-sectional as of the longitudinal muscle layer from one ein to another and this is indicated by the ge standard errors seen in these groups. No statistical difference could be shown between y of these groups. Denervation of veins from NCR was associated with a striking increase the average area of their longitudinal muscle layer The purpose of these expts. however s to compare denervated SHR and NCR, hence the lack of an appropriate sham-operated R group. That and the small numbers of observations precludes a definite conclusion th regard to this apparent effect.

The right-hand panel of Fig. 1 shows the values for maximum stress (force per unit area) ained in the various groups of portal veins in response to NA. Compared to NCR, both R and RHR veins develop much greater stress. Thus, the increased maximum contractile ce of SHR and RHR in comparison with NCR shown in Table I cannot be explained by increased effector tissue mass alone but reflects enhanced contractility of the smooth scle.

ooth muscle sensitivity and relatn responses to agonists in low concentrations of Ca^{2+}

e effector sensitivity t NA or ACh expressed as mean log ED_{50} values was determined om cumulative concentration-response relationships (see "Methods"). Fig. 2 illustrates ean log ED_{50} values obtained at normal (2.5 mM) and at several low Ca concentrations. he SHR portal ein displayed greater sensitivity to NA (Fig. 2 left panel) and to ACh ig. 2 right panel) at practically all Ca concentrations tested as seen from the log ED_{50} dues which range significantly below those of the NCR portal veins at most Ca levels. contrast, the aortic strips from the same groups of animals (Fig. . left panel) showed that e smooth muscle of this elastic artery from SHR was less sensitive to NA than that from CR over the same range of Ca^{2+} concentrations. In portal vein preparations from SHR, d NCR, the mean NA log ED_{50} values were not significantly different except at a Ca^{2+} ncentration of 0.2 mM which was the lowest one tested (5.28 ±0.04 and 5.11 ±0.05 (M) ean S.E., n 6, respectively) and during the second exposure to normal 2.5 mM calcium concentration (6.28 ±0.08 and 6.02 ±0.05 (M), respectively). Furthermore, the ED_{50} lues for NA were entirely comparable in RHR and NCR portal veins at all Ca^{2+} levels.

Another way of examining the function of the portal vein from normotensive and hyper nsive rats in graded low concentrations of Ca is illustrated in Fig 3 The peak contraction plitude of the spontaneous activity (SA) or the mean force developed in response to each ncentration of NA during the initial concentration-effect curve in normal Ca concen- tions have been set at 100 per cent. The SA amplitude and the individual responses to NA the several low Ca^{2+} concentrations have then been expressed as percentages of their espective control values. It is apparent that the spontaneous activity which is myogenic in igin, is practically abolished in all preparations at Ca concentrations of 0.8 mM or less. esponses t exogenous NA, on the other hand, show a NA concentration dependent in- ease in the percentage remaining in low calcium. It may be noted that the maximum sponse at 0.8 mM and at 2.5 mM often exceeded 100 per cent This is probably due to the act that the maximum responses of both SHR and NCR tended to grow in the course of the

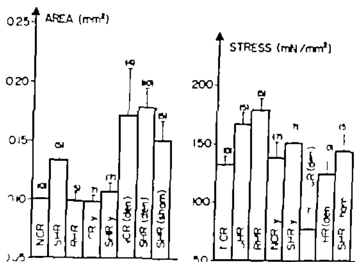


Fig. 1 *Left diagram.* Cross-sectional area of lo gitud nal media layer of portal eins from normotensn and several types of hypertensive rats. *Right diagram* Maximum stress in response to noradrenaline calculated from maximum f rce values (mN) given in Table I and cross-sectional area (left) Mean ±S E. Number of observations withi parentheses. indicates significant d fference (p 0 05) from ppropriate control group

aortic strips from 3 NCR_y compared to strips from 3 SHR_y (6.8 ± 1 1 and 3 1 ± 0 4 mN mean ± S E. p = 0 03).

Cross-sectional area and maximum stress

The maximum force developed in response to NA was related to the cross-sectional area of the longitudinal muscle of the portal vein as determined by light microscopy in order to obtain a measure of maximum stress (mN/mm²) in the individual tissues.

Preliminary studies showed that within the segment used the longitudinal muscle layer of the portal vein is reasonably uniform in as much as the maximum force developed was not altered by shortening the tissue. This was demonstrated by suspending a vein in an organ bath and measuring the maximum force developed in response to NA, 10 μM Then the vein was tied at the mid-point, cut and resuspended at the same passive force so that the length of tissue exerting force was reduced to one half the previous length The length of tissue could be reduced several times in this manner without altering maximum force induced by NA. Therefore it seemed reasonable to measure cross-sectional area of longitudinal muscle at the mid point of each tissue and relate it to the maximum force developed by the portal veins from NCR, SHR, RHR, NCR_y, SHR_y denervated SHR denervated NCR and sham-operated SHR

The results of these studies are shown in Fig. 1 In the left-hand panel it can be seen that the area of the longitudinal muscle layer of portal veins from SHR was greater than that of NCR controls, whereas veins from RHR were not different from NCR in this regard The average area of the longitudinal muscle layer in veins from SHR was slightly greater than that of NCR_y but was not statistically different.

Denervated veins from SHR were examined to see whether chronic sympathectomy would alter the mass of effector tissue compared to sham-operated veins from SHR and

ated veins from NCR due to elimination of any trophic influence exerted by the otor nerves. Surgery on the portal vein led to an increased variability of cross-sectional of the longitudinal muscle layer from one vein to another and this is indicated by the tandard errors seen in these groups. No statistical difference could be shown between these groups. Denervation of veins from NCR was associated with a striking increase average area of their longitudinal muscle layer. The purpose of these expts. however compare denervated SHR and NCR, hence the lack of an appropriate sham-operated group. That and the small numbers of observations precludes a definite conclusion egard to this apparent effect.

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h muscle sensitivity and relative responses to agonists in low concentrations of Ca^{2+}

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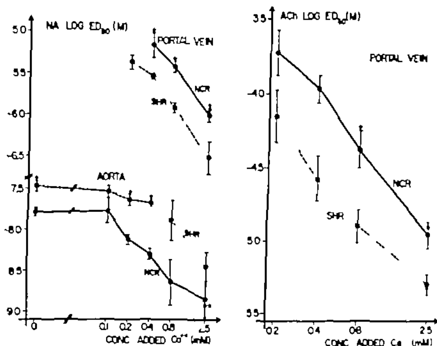


Fig. 2. Mean log ED_{50} values ($\pm$ S.E. n = 5) to noradrenaline (NA) determined in portal em preparatio and ortic strips (A) and mean log ED_{50} values ($\pm$ S.E.) t acetylcholine (ACh) determined n portal ve preparations (B) from normotensive (NCR) and spontaneoulsy hypertensive rats (SHR) at ormal Ca concentration (2.5 mM) and at several low Ca concentrations. Square symbols illustrate mea log ED values determined in 2.5 mM calci m tow rds end of experiment (See Methods for protocol). denot $p < 0.05$ Note that SHR portal vein is more sensitive to NA than NCR portal ei t all Ca levels here the opposite applies to the aortas and that portal vein from SHR is s gnificantly more sensit e than NCR ACh at most Ca calcium concentrat ons.

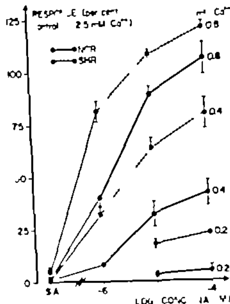
experiment as reflected by the observation that the final maximum response to NA in th last (second) exposure in 2.5 mM calcium was almost always greater than the maximum N response at the same Ca concentration at the beginning of the expt

In the adult SHR the residual responses to NA in low Ca concentrations were greate than those of NCR whereas no such difference occurred between SHR_y and NCR no between RHR and NCR. When ACh was the agonist the relative remaining response also were of comparable magnitude in SHR and NCR

Aortic strips from SHR were found to retain a smaller percentage of their control NA response than did NCR strips at all Ca concentrations studied Actually the per cent values at 0 4 and 0 8 mM Ca for responses of the NCR to NA at levels ranging from 1 nM to 10 μM all exceeded 50 per cent and did not show much variation with agonist concentration. Thus the aortic and portal vein smooth muscles seem to handle calcium quite differently and the dependence on external Ca for responses to NA is altered in opposite directions in these two tissues from SHR compared to NCR.

Discussion

Our results show that portal veins from hypertensive rats are functionally different from those of matched normotensive Wistar rats which served as controls. In all hypertensive



Contractile responses of portal vein from ...nsive rats (NCR solid line) and from spon... hypertensive rats (SHR interrupted line) ...rine (NA) at graded concentrations of ...mbers symbols indicate amplitude of spon... activity (SA). Responses expressed as ... of control responses to corresponding ...centrations determined during initial dose-... curve in 2.5 mM Ca²⁺ concentration. Mean ... Note that spontaneous activity dis... low calcium and that the residual responses ...are maintained in ... dose dependent manner

..., i.e. SHR, SHR_y and RHR, the portal vein preparation had increased maximum ...ses to agonists compared to controls. The aortic strips from SHR, however showed ...posite: decreased maxima, when compared to strips from NCR. Several workers have ...usly reported the decreased maximum responses to agonists in aortic strips from SHR ...er et al 1969 Clineschmidt et al. 1970, Shibata, Kurahashi and Kuchii 1973). Green-... and Bohr (1975) reported findings similar to ours in portal veins from SHR but the ...mum responses of portal veins from SHR_y and RHR have not been described previously ...r knowledge. Clineschmidt et al (1970) demonstrated that the maximum response of ... strips ...aried from one strain of rat to the other and pointed out the need for proper ...ols. The Wistar strain used in the present expts represents the most appropriate nor...nsive animal for comparisons to SHR which is available to us at present. Nevertheless, ...is an element of uncertainty present when quantitative comparisons hav... to be made ...een animals of different families. However the fact that RHR portal veins show in...ed maximum responses to agonists and increased spontaneous activity compared to ...—which were of identical strain—lends support to the tenet that portal veins from ...ertensive animals are indeed different from those of control animals. The observation ... aortic strips had decreased maxima while the portal vein from the same SHR had ...eased maxima as compared to control indicates interesting regional variation in the ...perties of vascular smooth muscle of the hypertensive animal.

...he increased maximum response in SHR portal veins is associated with both increased ...e mass of the longitudinal muscle and increased stress (force per unit area) whereas ...tal veins from RHR develop increased stress i... response to agonists but are not hyper...phied. Since the portal vein is not directly exposed to the increased arterial blood pressure, ... suggests that local hypertension per se is not responsible for the altered properties of

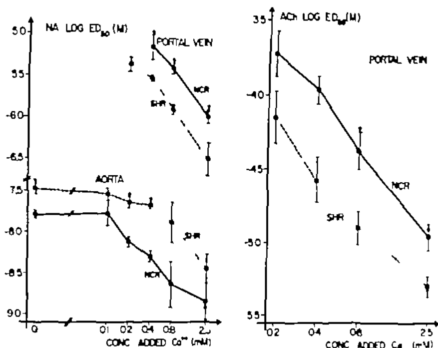


Fig 2. Mean log ED_{50} values (± S E. = 5) to noradrenaline (NA) determined in portal vein preparations and aortic strips (A) and mean log ED_{50} values (± S.E.) to acetylcholine (ACh) determined in portal vein preparations (B) from normotensive (NCR) and spontaneously hypertensive rats (SHR) at normal Ca concentration (2.5 mM) and at several low Ca concentrations. Square symbols illustrate mean log ED_{50} values determined in 2.5 mM calcium towards end of experiment (See Methods for protocol). denotes $p < 0.05$. Note that SHR portal vein is more sensitive to NA than NCR portal vein at all Ca levels whereas the opposite applies to the aortas and that portal vein from SHR is significantly more sensitive than NCR to ACh at most Ca calcium concentrations

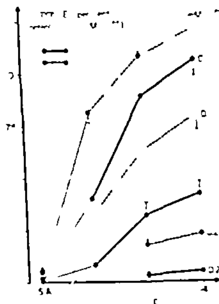
experiment as reflected by the observation that the final maximum response to NA in the last (second) exposure in 2.5 mM calcium was almost always greater than the maximum NA response at the same Ca^{++} concentration at the beginning of the expt

In the adult SHR the residual responses to NA in low Ca concentrations were greater than those of NCR whereas no such difference occurred between SHR and NCR_y nor between RHR and NCR. When ACh was the agonist the relative remaining responses also were of comparable magnitude in SHR and NCR

Aortic strips from SHR were found to retain a smaller percentage of their control NA response than did NCR strips at all Ca concentrations studied Actually the per cent values at 0 4 and 0 8 mM Ca for responses of the NCR to NA at levels ranging from 1 nM to 10 µM all exceeded 50 per cent and did not show much variation with agonist concentration. Thus the aortic and portal vein smooth muscles seem to handle calcium quite differently and the dependence on external Ca for responses to NA is altered in opposite directions in these two tissues from SHR compared to NCR

Discussion

Our results show that portal veins from hypertensive rats are functionally different from those of matched normotensive Wistar rats which served as controls. In all hypertensive



1. Contractile responses of portal vein from [illegible] rats (NCR solid line) and from spontaneously hypertensive rats (SHR interrupted line) [illegible] (NA) at graded concentrations of [illegible] Square symbols indicate amplitude of spontaneous activity (S.A.). Responses expressed [illegible] of control responses to corresponding concentrations determined during initial dose response curve in 2.5 mM Ca concentration. Mean ± S.E. [illegible] 5. Note that spontaneous activity [illegible] low calcium and that the residual responses [to] NA are maintained in a dose dependent manner.

[illegible] SHR, SHR_y and RHR, the portal vein preparation had increased maximum [re]sponses to agonists compared to controls. The aortic strips from SHR, however, showed [th]e opposite: decreased maxima, when compared to strips from NCR. Several workers have [pre]viously reported the decreased maximum responses to agonist in aortic strips from SHR [(Sp]ector *et al.* 1969; Clineschmidt *et al.* 1970; Shibata, Kurahashi and Kuchii 1973). Green[be]rg and Bohr (1975) reported findings similar to ours in portal veins from SHR but the [ma]ximum responses of portal veins from SHR_y and RHR have not been described previously [to] our knowledge. Clineschmidt *et al.* (1970) demonstrated that the maximum response of [ao]rtic strips varied from one strain of rat to the other and pointed out the need for proper [con]trols. The Wistar strain used in the present expts. represents the most appropriate nor[mo]tensive animal for comparisons to SHR which is available to us at present. Nevertheless, [the]re is an element of uncertainty present when quantitative comparisons have to be made [be]tween animals of different families. However, the fact that RHR portal veins show in[c]reased maximum responses to agonists and increased spontaneous activity compared to [N]CR—which were of identical strain—lends support to the tenet that portal veins from [h]ypertensive animals are indeed different from those of control animals. The observation [th]at aortic strips had decreased maxima while the portal vein from the same SHR had [in]creased maxima as compared to control indicates interesting regional variation in the [p]roperties of vascular smooth muscle of the hypertensive animal.

The increased maximum response in SHR portal veins is associated with both increased [t]issue mass of the longitudinal muscle and increased stress (force per unit area) whereas portal veins from RHR develop increased stress in response to agonists but are not hyper[t]rophied. Since the portal vein is not directly exposed to the increased arterial blood pressure, this suggests that local hypertension *per se* is not responsible for the altered properties of

ceptors. The portal veins from RHR did not display increased agonist sensitivity. This suggests that the mechanisms involved in the increased portal vein responsiveness in RHR are different from those of SHR.

Hallbäck, Lundgren and Weiss (1971) observed no difference in NA ED_{50} values in portal veins from SHR and NCR. Most methodological aspects were the same in their study except for two points. We calculated mean log ED_{50} values (Fleming *et al.* 1975) from cumulative concentration-response curves whereas they used arithmetic means and non-cumulative concentration-response curves. It is interesting to note that desensitization to NA (but not ACh) occurred in our expts. The second concentration-response curve in normal Ca concentration for the portal vein from SHR gave a higher ED_{50} value than the first one (Fig. 7). In fact, the second ED_{50} in normal Ca in the SHR and NCR portal vein did not differ significantly one from the other. In control experiments it was found that the desensitization in the SHR was due to the repeated exposure of the tissue to NA and not simply the passage of time or exposure to altered calcium concentration (Sutter and Ljung, unpublished). Thus, it did occur on repeated exposure to NA at a constant Ca concentration 2.5 mM. It did not occur when the same period of time was simply allowed to elapse between one initial and one final dose-response curve to NA without intervening exposures to NA. It is also interesting to note that the ED_{50} to NA in 0.8 mM calcium was different in SHR compared to NCR portal veins (Fig.) even though this ED_{50} determination was very close in time to the second ED_{50} in normal calcium where no difference in ED_{50}'s was seen. It seems that the stress of lowered external calcium promoted detection of the differing ED_{50}'s of the SHR compared to the NCR portal vein.

Another difference among the portal veins from hypertensive rats involved their handling of calcium. Portal veins from SHR retained their responses to NA in low calcium better than those from NCR. Veins from SHR_{Y} and RHR were not different from controls in this regard. Similar results have been found in perfused rat hindquarters (Folkow *et al.* 1977 and unpublished), where SHR were found to retain their pressor responses to NA in low calcium better than controls but SHR_{Y} only minimally showed this effect and RHR not at all. We found no consistent difference in per cent response retained in low calcium when SHR veins were compared to NCR veins with ACh as the agonist. This may indicate a variation in the effects of NA and ACh on calcium kinetics in the portal vein, as previously shown for cutaneous veins based on differential blockade of the responses to NA and ACh by verapamil (Shepherd and Vanhoutte 1975).

Finally one must ask what could cause the structure and function of a vessel like the portal vein to be altered in hypertensive rats? There have been reports of decreased compliance of femoral and mesenteric veins in rats and *in situ* (Overbeck 1972, Simon *et al.* 1975) in perinephritic dogs. These authors concluded that structural alterations of the veins were involved. Simon (1976) has reported that SHR also shows decreased venous compliance in vivo. Bevan *et al.* (1975 a, b, and 1976) have reported that veins from rabbits made hypertensive by partial constriction of the aorta proximal to the renal arteries show increased responsiveness to NA and to nerve stimulation which they attribute to an increased sensitivity of α-adrenergic receptors. The physiology of the portal vein which we used is quite unlike the saphenous and cephalic veins these latter workers studied, so that their

the portal vein At present it cannot be ruled out completely that the transmural pressure of the portal vein is in fact elevated in hypertension. However the difference between RHR and SHR with regard to cross-sectional area seems to indicate that in SHR as opposed to RHR there may be a systemic factor relatively independent of pressure which produces hypertrophy Greenberg and Bohr (1975) did not find hypertrophy in their study of portal veins from SHR but they measured cross-sectional area by dividing weight by length. This technique fails to separate out longitudinal from circular muscle and connective tissue while the histological method we used has the merit of measuring only the longitudinal muscle layer It is this latter muscle alone which is involved in development of force in the suspended vein. It should be pointed out that light microscopy in turn, has the limitation of neither providing information about the actual muscle cell mass nor the absolute amount of contractile filaments of the transverse section of the longitudinal muscle layer Consequently it is possible that the increased stress values, based on cross-sectional areas determined by light microscopy in portal veins from hypertensive animals may reflect increased relative mass of contractile elements.

Trophic influences from the sympathetic vasomotor fiber supply of the portal vein might be one factor which induces and maintains the hypertrophy of the SHR portal vein. Our attempt to analyze whether chronic denervation would eliminate the differences between SHR and NCR portal veins was complicated by the surgery apparently increasing the variability of response from one vein to another It seems clear however that denervation did not reduce the increased maximum response to agonists nor did it alter the hypertrophy of portal veins from SHR If anything denervation produced increased cross-sectional area in veins from NCR and decreased their maximum responses. It may be that a week or 10 days was insufficient time for the effects of denervation to be seen but no longer time was allowed because of this risk of re-innervation and development of postjunctional supersensitivity It might be that denervation not only of the portal vein but also of the arterial resistance vessels of the splanchnic region caused increased transmural pressure in the portal vein. Thus the surgical procedure would not only eliminate a possible trophic influence but could cause hypertrophy of the myogenically active smooth muscle

Clear differences both in structure and function exist among the portal veins from the several hypertensive groups of rats. The lack of hypertrophy in veins from SHR and RHR has already been mentioned. An additional difference involves the sensitivity to agonists. Portal veins from adult SHR showed lower ED_{50} values for both NA and ACh than did NCR portal veins at all calcium concentrations studied In the portal veins from young rats the NA ED_{50} value was significantly lower in SHR compared to NCR only at 0.2 mM Ca^{2+} concentration. This suggests that the increased agonist sensitivity in SHR veins is part of an ongoing process during the development of hypertension Thus, at the age of 6-8 weeks (SHR_y) it is demonstrable only upon provocation by the low calcium concentration. Adrenergic receptor stimulation was not involved in the determination of ACh concentration effect curves, because phentolamine, in concentrations which blocked 90 per cent of the response to a maximum dose of NA, was present in these experiments. The fact that the ED_{50} values to both NA and ACh were lower in SHR than in NCR portal veins means that the mechanisms responsible for the increased sensitivity in SHR are not limited to adrenergic

[illegible], S and D F Bohr, Venous smooth muscle in hypertension [illegible] of portal vein from spontaneously hypertensive rat *Circulat Res* 1975 *36*/*37* Suppl I [illegible]

[illegible], M Interaction between central neurogenic mechanisms and [illegible] vascular design in primary hypertension. Experimental studies in spontaneously hypertensive rats *Acta physiol scand* 1975 Suppl 424

[illegible], M., Y Lundgren and L Weiss, Reactivity to noradrenaline of aortic strips and portal veins from spontaneously hypertensive and normotensive rats *Acta physiol scand* 1971 *81* 176-181

[illegible], P M and G B Weiss, Differential effect of calcium removal upon vascular smooth muscle contraction induced by norepinephrine, histamine and potassium *J Pharmacol exp Ther* 19[illegible] *1*[illegible] [illegible]

[illegible], B, B Ljung, T Malmfors and L Olson, Prejunctional supersensitivity in rat portal vein related to its pattern of innervation *Acta physiol scand* 1970 Suppl 349 5 [illegible]

[illegible], B, *Physiological patterns of neuroeffector control mechanisms* [illegible] Symp Vascular Neuroeffector Mechanisms, Odense, 1975 S Karger AG.

[illegible], Y Adaptive changes of cardiovascular design in spontaneous and renal hypertension. Hemodynamic studies in rats *Acta physiol scand* 1974 Suppl 40[illegible]

[illegible] M J and W Halpern, Evidence for increased contractility in primary [illegible] secondary resistance vessels [illegible] spontaneously hypertensive rats *Acta physiol scand* 1976 Suppl 440 Abs [illegible] 182

[illegible], H W Hemodynamics of early experimental renal hypertension in dogs, normal limb blood flow, elevated limb vascular resistance, and decreased venous compliance *Circulat Res* 197[illegible] *31* 653-663

[illegible], B L and M C Sutter, Vasomotion and contraction of the perfused mesenteric portal vein. Effect of drugs and altered perfusion pressure *Can J Physiol Pharmacol* 1971 *49* 615-618

[illegible], J T and P M Vanhoutte, *Veins and their control* [illegible] W B Saunders Company Ltd London 1975

[illegible], S K Kurahashi and M K [illegible], A possible etiology of contractility impairment of vascular smooth muscle from spontaneously hypertensive rats *J Pharmacol exp Ther* 1973 *185* 406-417

[illegible], G, Altered venous function in hypertensive rat *Circulat Res* 1976 *39* 412-418

[illegible], G M B P[illegible], J F Dunne and H W Overbeck, Mesenteric hemodynamics [illegible] early experimental renal hypertension in dogs. *Circulat Res* 1975 *36* 791-798

[illegible], S J, H Fuenza, H M Maling and B B Burow Vascular smooth muscle reactivity in normotensive and hypertensive rats *Science* 1969 *164* 1300-1301

results may have limited relevance to the present study Taken together however all these reports indicate that vascular changes in the hypertensive state are not limited to arteries. It is entirely possible that slightly elevated transmural pressures may operate on the venous side in hypertensive states and thus induce secondary changes. Furthermore, humoral and neurogenic influences in hypertension would be expected to affect arterial *and* venous muscle.

Our results demonstrate that portal veins from hypertensive animals are different from veins from normotensive animals. We indicated in the Introduction that the portal vein in several functional respects resembles the true resistance vessels, for example in its basic physiology and in dependence on extracellular calcium ion concentration (Rhodes and Sutter 1971 Folkow *et al* 1977 Ljung 1977) Thus in the hypertensive state it may well be affected by generalized factors in a manner similar to resistance vessels. If this is so, our results also indicate the following: the hypertrophy seen in arteries of perfused hindquarters of SHR (Folkow *et al* 1973) would not entirely be due to high arterial pressure but may involve a systemic, perhaps genetic tendency of vascular smooth muscle to hypertrophy in these animals increased maximum contractility is involved in the higher arterial pressure observed in SHR and their vascular smooth muscle seems to handle calcium differently from controls the mechanism of hypertension in RHR is different from that in SHR but again involves increased maximum responses to agonists. The latter is independent both of vascular architecture and of adrenergic receptors and hence may involve more efficient coupling between stimulus and vascular smooth muscle contraction

We thank Miss Ann Kjellstedt for skilled technical assistance, Mr Lars Stage for computer programming and Miss Gerd Mellhage f secretarial aid This w k was supported by a B. C Heart Fo ndation special travel grant to M C. S. and by the Swedish Medical Research Council (3884 and 00016), and by AB Hässle

References

BEVAN J A., R D BEV N, P C CHANG, B L PEGRAM, R E. PURDY and CHI SU Cha ges the contractile response of arteries nd veins from hypertensive rabbits t ympathetic nerv activity Assessment of some postsynaptic influences. *Blood Vessels* 1976. *13* 167 180.

BEVAN, J A., R D BEVAN, P C. CHANG, B L PEGRAM, R E PURDY and CHI SU Analysis of changes in reactivity of rabbit arteries and veins two weeks after induction of hypertension by coarctation of the abdominal aorta *Circulat R s.* 1975 *37* 183–190.

BEVAN R D R. E. PURDY CHI SU and J A. BEV N, Evidence for increase n adrenergic nerve function in blood vessels from experimental hypertensive rabbits *Circulat R* 1975 b *37* 503–508

CLINESCHMIDT B. V R G GELLER, W C GOVIE and A SJOERDSMA, Reactivity to norepinephrine and nature of the alpha adrenergic recepto in vascula smooth muscle of a genetically hypertensive rat. *Eur J Pharmacol* 1970. *10* 45–50

COLLINS, G A., M C. SUTTER and J C TEISER, Calcium and contraction n the rabbit anterior mesenteric portal vein. *Can J Physiol Pharmacol.* 1972. *50* 289–299

FINCH L. and G HAEUSLER, Vascular resistance a d reactivity hypertensive rat *Blood Vessels* 1974. *11* 145–158.

FLEMING, W W D P WESTFALL, I S. de la LANDE a d L. B JELLETT Log-normal distribution of equi-effective doses of norepinephrine and acetylchol ne i several tissues. *J Pharmacol p Ther* 1972 *181* 339–345

FOLKOW B M. HALLBÄCK, J JONES and M C. SUTTE Effects of noradrenaline on consecutiv vascular segments at low or normal calcium concentrations NCR and SHR. *Clin. Sci Mol Med.* 1977 I press

FOLKOW B M HALLBÄCK, Y LUNDGREN, R. SIVE TSSON nd L WEISS, Importance of adaptive changes in vascular design for establishment of primary hypertension, studied in ma and i spontaneously hypertensive rats. *Circulat Res* 1973 *32–33* Suppl. I I 2 13

...cannula inserted into the corporal region of the stomach. Since the ... were lying on their ... the gastric juice left the gastric cavity via the cannula. Gastric juice samples were collected every ... and titrated for their acid content with 0.01 M NaOH. Phenolphthalein was used as indicator. ... vagal nerves were cut in the cervical region. The distal ends were stimulated electrically ... 5 ms duration and ... 10 Hz. Effect on heart rate and blood pressure was taken as evidence of ... vagal stimulation.

... which the gastrin output ... determined quantitatively, additional surgical procedures were ... The arteries below the duodenum, and the spleen were taken ... The remaining portal ... consequently composed of blood draining the stomach, but also of some duodenal and pancreas ... thus referred to as gastric blood. The portal vein was ligated close to the liver and the blood end of the ... mesenteric vein was cannulated. Thus the gastric blood was conveyed back ... out of the ... drop chamber unit, allowing continuous recording of the rate of bloodflow and ... then led ... into the cat through ... femoral vein.

... blood samples were collected from the gastric blood and gastrin determinations were ... by radioimmunoassay (Nilsson 1975). The gastrin output ... was calculated as the product of ... concentration and rate of gastric plasma flow. The experiments in which gastric acid secretion ... was studied, were differently designed.

... of atropine and methamide on vagally induced gastric acid secretion

... stimulations were performed with 2–10 Hz for 1–3 h. Atropine (0.1–0.3 mg/kg) or methamide 4 ... 20 mg/kg was given, when the secretory response seemed to have reached a plateau (30–40 min ... stimulation was begun). In each experiment the mean acid output following two consecutive 7.5 min ... was calculated for the last 15 min period before and the two first 15 min period after administration of ... the drugs. The amount of HCl secreted per 15 min after the drugs had been given, was then ex... ... per cent of the amount secreted during the first 15 min.

... effect of atropine and methamide on vagally induced gastrin release

... stimulations were performed at 2 and 5 Hz for 5 or 10 min. Two identical stimulations were performed ... Atropine 0.1 ... mg/kg or methamide 20 mg/kg ... given *15 min after* the first and *10 min* ... the second stimulation. The gastrin output following the two stimulations was calculated and the ... induced by the second stimulation was expressed in per cent of the amount of gastrin released by ... stimulation.

... following drugs were used: Atropine sulphate (Atropin, ACO), Methamide (Smith, Kline and ... laboratories Ltd.).

Results

... rate of HCl secretion induced by vagal stimulation with 2–10 Hz was clearly reduced ... atropine in doses of 0.1–0.3 mg/kg (n = 6) (Fig. 1). The gastric juice collected between ... and 15–30 min after atropinization averaged ~40% and ~30% of the amounts se... ... during the control period (Table 1 a).

... effect of methamide on vagally induced gastric secretion was tested in doses of 4 ... and 20 mg/kg (n = 3). The rate of gastric juice secretion was ~30 and 10% respectively ... the first and second 15-min period following administration of methamide (Fig. 2, ... 1 b).

... the other hand, the gastrin release response to vagal stimulation at 2 and 5 Hz was not ... affected by atropine in doses varying from 0.2–2 mg/kg (n = 10, Table II b, Fig. 3). Ad... ...istration of atropine did in fact lead to a slight increase in the vagally induced gastrin ... output (150%). The corresponding value, when no drugs were given was ~110% (n = 5 ... Table II a. Methamide 20 mg/kg did not reduce the gastrin output following vagal stimula... (n = 4) (Fig. 4).

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Effect of Atropine and Methiamide on Vagally Induced Gastric Acid Secretion and Gastrin Release in Anesthetized Cats

By

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Abstract

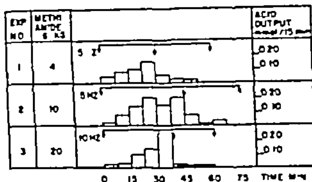
UVNÄS-WALLENSTEN, K. and H. ANDERSSON. *Effect of atropine and methiamide on vagally induced gastric acid secretion and gastrin release in anesthetized cats.* Acta physiol. scand. 1977. 99. 496–502.

Gastrin release and HCl secretion was induced by electrical vagal stimulation in anesthetized cats. Both atropine (0.1–0.3 mg/kg) and methiamide (4–70 mg/kg) inhibited the HCl secretion (by ~60 and ~80 %). On the other hand, neither atropine (0.2 mg/kg) nor methiamide (20 mg/kg) inhibited the vagally induced gastrin release. Possible explanations of the atropine resistance of the vagal gastrin release mechanism are discussed.

In the cat, electrical vagal stimulation induced a release of gastrin into the gastric venous outflow and also initiated a secretion of gastric juice (Uvnäs *et al.* 1975 and Uvnäs-Wallensten *et al.* 1976). However, while the secretion of gastric juice continued as long as the stimulations were performed, the antral gastrin output declined after an initial peak and basal gastrin levels were reached again within 15–20 min. This difference between the two vagal responses indicates, that the nervous effector mechanisms behind these effects may be dissimilar. On continuous vagal stimulation with less than 2–3 000 impulses the gastrin output per impulse was constant. This feature of the gastrin release mechanism makes the cat preparation well suited for quantitative testing of the influence of substances on vagally induced gastrin release. The present study was performed to investigate to which extent atropine and methiamide antagonize gastric acid secretion induced by electrical vagal stimulation directly at the parietal cell level or via a diminished release of gastrin.

Methods

The experiments were performed on cats anesthetized by chloralose (50 mg/kg) and urethane (100 mg/kg). The cats weighed between 2–4 kg and had been deprived of food for 18 h. Blood pressure, pulse rate and temperature were continuously recorded and the cats were artificially ventilated. The cats were provided



. 3 expts. demonstrating the ...itory effect of methiamide on ...ally induced HCl secretion. ...al stimulations ...ere performed 5 and 10 Hz. Methiamide ... mg kg ... as administered ...n the HCl secretion had ...ched plateau level (see also ...le I b).

...le II and II b. Two successive ...gal stimulations ...ere performed ... 2 or 5 H ... for 10 min in 5 ... ts (II a). In 10 other cats atropine in doses ranging from 0 ... 2 mg kg ... ere given 10 min before the second stimulation (2 or 5 Hz) (T... ble II b). The gastrin output following the second stimulation was expressed as per cent of the preceding control stimulation. The interstimulatory period was 20-30 min.

...le II

...t no.	Frequency Hz	Gastrin release induced by the second stimulation in per cent of the control stimulation (... 100 ...)
	5	115
	5	95
	5	103
	5	119
	5	115

...ean 110 per cent.

...le II b

...t no.	Frequency Hz	Atropine mg kg	Gastrin release induced by the second stimulation in per cent of control stimulation (= 100 ...)	
6	2	0 2	72	
7	2	0 3	97	
8	2	0 3	440	
9	2	0 6	185	
0	2	2 0	103	180
1	5	0 25	152	
2	5	0.25	83	
3	5	0.5	134	
4	5	1 0	158	
5	5	2 0	103	126

Mean 153 per cent.

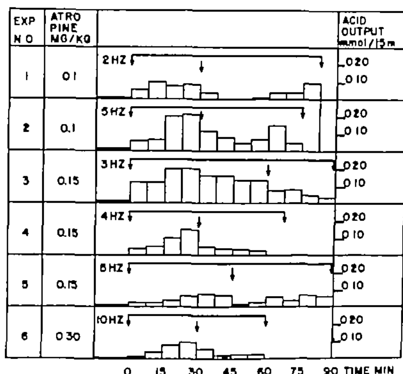


Fig. 1 6 expts. demonstrati g the nhibitory effect of tropine on vagally induced HCl secretion. Vaga stimulations were performed at 2 10 Hz. Atropine 0 1–0.3 mg/kg was adm istered when the HCl secretion had reached a platea level and is ndicated by an rrow in each expt (see also Table I a).

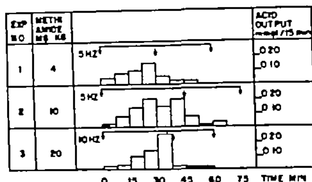
TABLE I and I b *Effect of tropine (0.1–0.3 mg/kg) (Table I a) and Meth amide (4–20 mg/kg) (Table I b) on vagally induced HCl secretion.* A – Acid output 15 m before, B 0–15 m and C 15–30 m after the drugs were given. The secretion during periods B and C has been calculated in per cent of the co trol period prio t dministration of atropine and methiamide (see also F g 1 nd 2)

TABLE I a

Exp. no	Atropine mg/kg	A	B		C	
		mmol	mmol	per cent of A	mmol	per cent of A
1	0.1	0.09	0.0?	22	0 01	11
2	0.1	0.21	0 10	48	0.06	?9
3	0.15	0.14	0 08	57	0 05	36
4	0.15	0 13	0 05	38	0 03	23
5	0.15	0.07	0.03	43	0 05	71
6	0.3	0.09	0.05	56	0 02	22

TABLE I b

Exp no.	Methiamide mg/kg	A	B		C	
		mmol	mmol	per cent of A	mmol	per cent of A
1	4	0.13	0 05	38	0 0.	15
2	10	0.18	0 05	28	0 03	17
3	?0	0 2	0.03	15	0 01	5



] ... demonstrating the ...y effect of methiamide on ... induced HCl secretion ... stimulations were performed ... and 10 Hz. Methiamide ... mg/kg ... as administered ... the HCl secretion had ... plateau level (see also ...)

II and II b. Two successive vagal stimulations were performed at 2 or 5 Hz for 10 min in 5 cats (II a). In 10 other cats atropine in doses ranging from 0 ... mg/kg were given 10 min before the second stimulation (2 or 5 Hz) (Table II b). The gastrin output following the second stimulation was expressed in per cent of the preceding control stimulation. The interstimulatory period was 20-30 min.

II

	Frequency Hz	Gastrin release induced by the second stimulation in per cent of the control stimulation (= 100)
	5	115
	5	95
	5	103
	5	119
	5	115

110 per cent.

II b

	Frequency Hz	Atropine mg/kg	Gastrin release induced by the second stimulation in per cent of control stimulation (= 100)	
	2	0	72	
	2	0.5	97	
	2	0.5	440	
	2	0.6	185	
	2	2.0	183	190
	5	0.25	152	
	5	0.25	83	
	5	0.5	134	
	5	1.0	158	
	5	2.0	103	126

153 per cent.

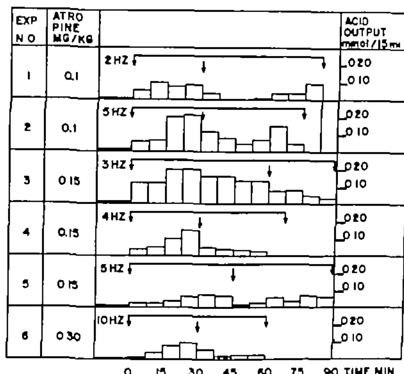


Fig. 1 6 expts. demonstrating the i hibitory effect of tropine on vagally oduced HCl secretion V. stimulat ons were performed at 2 10 Hz. Atropine 0.1–0.3 mg/kg was dministered when the HCl secre had reached a platea level and is ndicated by an arrow each expt. (see also Table I a).

TABLE I a and I b *Effect of tropine (0.1–0.3 mg/kg) (Table I) and Methiamide (4–20 mg/kg) (T ble* on vagally induced HCl secretion A = Acid output 15 min before, B 0–15 min and C 15–30 after the drugs were given. The secretio during periods B and C has been calculated per c of the control period prior to administration of atropine and methiamide (see lso Fig 1 and

TABLE I

Exp. no.	Atropine mg/kg	A	B		C	
		mmol	mmol	per cent of A	mmol	per cent of A
1	0 1	0.09	0.0..	..	0 01	11
2	0 1	0..1	0 10	48	0.06	29
3	0.15	0.14	0 08	57	0 05	36
4	0.15	0.13	0 05	38	0 03	..3
5	0.15	0 07	0 03	43	0 05	71
6	0.3	0 09	0.05	56	0.0	22

TABLE I b

Exp no.	Methiamide mg/kg	A	B		C	
		mmol	mmol	per cent of A	mmol	per cent of A
1	4	0.13	0 05	38	0 02	15
2	10	0.18	0 05	..8	0 03	17
3	20	0.2	0 03	15	0 01	5

Jhon et al. 1972, Tepperman et al. 1975), it was surprising to find that in the present periments atropine (0.2–2 mg/kg) was without effect on the vagally induced gastrin release. The results are at variance with those obtained in sham feeding experiment (Nilsson et al. 1972 and Tepperman et al. 1972). In those expts. both gastrin release and HCl secretion were blocked by 0.2 mg/kg of atropine. However sham feeding induces vagal activation indirectly and most certainly several central neural pathways, which might be atropine sensitive are involved. Thus, since atropine passes the blood brain barrier the inhibiting effect of atropine on gastrin release is not necessarily exerted peripherally at the gastrin cell level. Species differences behind the atropine resistance are less probable, since Smith et al (1975) failed to block vagally induced gastrin release in dog expts. similar to ours. Furthermore, the gastrin release induced by sham feeding in dogs is not abolished by a quarternary anticholinergic, which does not penetrate the blood brain barrier (Uvnäs-Wallensten and Andersson, to be published).

Theoretically the administration of atropine might have caused higher antral pH during the period of vagally induced gastrin release through its inhibitory effect on HCl secretion. An atropine induced reduction of the gastrin release might in that case be masked by a simultaneous facilitation of the gastrin release by a higher antral pH. However the vagally induced gastrin peak appears within a few minutes after initiation of stimulation, when only insignificant amounts of HCl may have been produced, rendering such a hypothesis unlikely.

The inability of atropine to inhibit the vagally induced gastrin release might be explained in different ways. Either the vagi contain non-cholinergic neurons, which mediate the gastrin response or the fibres are cholinergic, but the receptors on the gastrin cells are not of the traditional atropine sensitive muscarinic type.

Other parasympathetic effects have been demonstrated to be atropine insensitive. Martinson (1972) studied the effect of electrical vagal stimulation on gastric motility and tone. At certain stimulatory parameters a relaxation of the stomach was induced, which was only partly blocked by atropine, even when given in very high doses. Since the gastric relaxation was abolished when small amounts of ganglionic blockers were added, it was suggested that the partial inhibitory effect of atropine on the vagally induced gastric relaxation was exerted at the ganglionic level.

Adrenergic fibres are present in the vagi (Lundberg et al. 1976) and adrenergic nerves have been demonstrated within the antral mucosa (Krokhina 1973). Adrenergic fibres could then be activated by electrical stimulation of the vagi. In humans, injections of adrenaline has been shown to induce a release of gastrin, which can be blocked by beta-adrenergic blocking substances (Stadil and Rehfeld 1973). However neither alpha nor beta-adrenergic blocking agents were able to inhibit the vagally induced gastrin release—rendering such an hypothesis unlikely (unpublished observations).

Since gastrin is released by antral perfusion with acetylcholine (Jackson et al. 1972) and also after i.v. infusion of this substance (Booth et al. 1974), the gastrin cells seem to be provided with receptors sensitive to acetylcholine. If these receptors correspond to the post ganglionic parasympathetic nerve terminals, they are not of the ordinary atropine sensitive muscarinic type. However acetylcholine is not only postganglionic transmittor substance,

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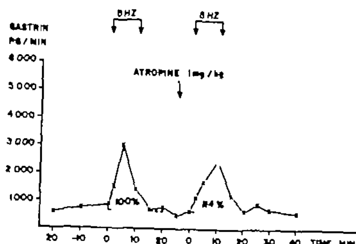


Fig. 3

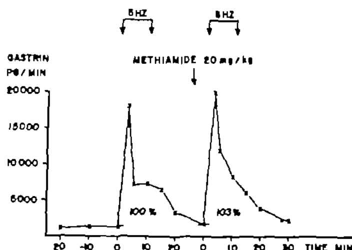


Fig. 4

Fig. 3 and 4 The experiment demonstrates the lack of effect of atropine (Fig 3) and methiamide (Fig 4 on vagally induced gastrin release. Two successive vagal stimulations were performed at 5 H for 10 min Atropine (1 mg/kg) or methiamide 20 mg/kg was given 10 min before the second stimulation.

Discussion

The well established inhibitory effect of atropine on vagally induced gastric acid secretion was confirmed by the present expts. Methiamide, a histamine-2 receptor antagonist was an even more potent inhibitor in the dosage range tested. Neither atropine, nor methiamide blocked the gastrin release caused by vagal stimulation In other words, neither of the drugs inhibit the gastric secretory response by inhibition of gastrin release.

Atropine (0 1–0 3 mg/kg) reduced the acid secretory response to vagal stimulation by more than 50%. Similar doses have been shown to almost abolish the HCl secretion induced by sham feeding in dogs (Nilsson *et al* 1972, Tepperman *et al* 1972)

Since vagally induced gastrin release is supposed to be mediated via cholinergic fibres

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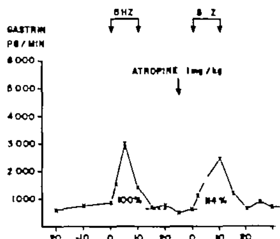


Fig. 3

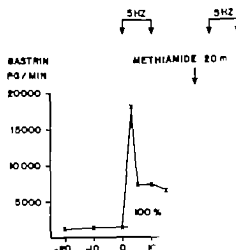


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Acta physiol. scand. 1977 99 503–509

From the Department of Pharmacology University of Göteborg, and from the Brain Research Laboratory E-blocket, University Hospital, Lund, Sweden

Effect of Hypercapnia and Hypocapnia on Tryptophan and Tyrosine Hydroxylation in Rat Brain

By

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Abstract

Carlsson, A T Holmin, M. Lindqvist and B. K. Siesjö. *Effect of hypercapnia and hypocapnia on tryptophan and tyrosine hydroxylation in rat brain*. Acta physiol. scand. 1977 99 503–509

The effects of induced hypo- and hypercapnia upon the rate of hydroxylation of tryptophan and tyrosine in the rat brain were studied by measuring the accumulation of 5-HTP and DOPA following administration of the aromatic L-aminoacid decarboxylase inhibitor 3-hydroxybenzylhydrazine HCl (NSD 1015). The results suggest that the hydroxylation of tryptophan varies directly with the tissue P_{O_2}. On the other hand, the hydroxylation of tyrosine did not show a simple relationship to P_{O_2} but appeared to be influenced by pH changes.

The rate-limiting enzymes in the synthesis of catechol and indole amines are tyrosine and tryptophan hydroxylase, respectively. These enzymes are oxygen dependent and their activities vary with the P_{O_2} *in vitro* (Fisher and Kaufman 1972, Green and Sawyer 1966). Recently it was shown that administration of gas mixtures with low oxygen content reduces the rate of hydroxylation of tyrosine and tryptophan *in vivo* (Davis and Carlsson 1973 a and b) and, for tryptophan, the rate of hydroxylation was found to change linearly with the cerebral venous P_{O_2} (Davis *et al.* 1973). The results suggest that the rate of synthesis of amines is related to tissue P_{O_2}. However hypoxia *in vivo* induces other changes in the tissue than a mere reduction of P_{O_2}. For example, it should be recalled that although the cerebral energy state is upheld at the oxygen tensions previously used to study monoamine metabolism, cellular acidosis develops at arterial P_{O_2} values below about 50 mmHg (Siesjo and Nilsson 1971 MacMillan and Siesjo 1972, Norberg and Siesjo 1975). Previous studies indicate that hypercapnia may reverse the effect of hypoxia on catecholamine but not on 5-hydroxytryptamine (5-HT) synthesis in the rat brain (Brown *et al.* 1974).

In the present experiments we have studied the effects of induced hypocapnia and hypercapnia upon the rate of hydroxylation of tryptophan and tyrosine in the rat brain, employing a technique in which the accumulation of 5-HTP and DOPA was measured following administration of the aromatic L-aminoacid decarboxylase inhibitor 3-hydroxybenzylhydrazine

it is also an intraganglionic transmittor Assuming that the gastrin cells serve as the post ganglionic neurons of endocrine parasympathetic nerves (in analogy with the noradrenaline and adrenaline containing cells in the adrenal medulla) the cholinergic receptors could be of the nicotinic type.

In conclusion the vagal neuro-effector junction at the antral G-cells differ from those on the HCl glands. Either the vagal fibres are not cholinergic—or they are cholinergic—but then the G-cell receptors are not of the muscarinic type.

The investigation has been supported by gra ts from Magnus Bergvalls Stiftelse

References

BOOTH, R. A. D D D REEDER and J C. THOMPSON Effect of intra enous acetylcholine on serum gastrin levels i dogs. *Proc Soc xp Biol* (N Y) 1974 *146* 1156–1158

JACKSON, M D D REEDER and J C THOMPSON Dynamic characteristics of gastrin release. *Amer J Surg* 1972. *123* 137 142.

KROKIUNA, E. M Sympathetic innervation of the gastrointestinal tract of mammals. *Arch Anat mikr Morph xp* 1973 *62*. 307 321

LUNDBERG, J H. AHLMAN, A. DAHLSTRÖM and J KEWENTER, Catecholamine-containing nerve fibres in the human abdominal vagus. *Gastroenterology* 1976. *70*. 472 774

MARTINSSON J Vagal relaxation of the stomach experimental reinvestigation of the concept of the transmission mechanism. *Acta physiol. scand.* 1965 *64* 453–462.

NILSSON, G Increased plasma gastrin levels in connection with inhibition of gastric acid responses to sham feeding following bulbar perfusion with acid in dogs. *Scand. J Gastroent* 1975 *10* ?73–277

NILSSON G J SIMON, S A BERSON and R. S. YALOW Plasma gastrin and gastric acid responses to sham feeding and feeding in dogs. *Gastroenterology* 1972. *63* 51 59

SMITH, C. L. I KEWENTER, A. M CONNELL, J ARDILL, R. HAYES and K BUCHAN N, Control factors in the release of gastrin by direct electrical stimulation of the vagus. *Dig Dis.* 1975 *20* 13–2.

STADIL, F and J F REHFELD Release of gastrin by epinephrine in man. *Gastroenterology* 1973. 65 10-215.

TEPPERMAN, B. L., J H WALSH and R. M. PRESHAW Effects of antral denervation on gastrin release by sham feeding and insulin hypoglycemia in dogs. *Gastroenterology* 1972. *63* 973–980

UVNÄS, B. K. UVNÄS-WALLENSTEN and G NILSSON Release of gastrin on vagal stimulation in the cat. *Acta physiol scand.* 1975. *94* 167 176.

UVNÄS-WALLENSTEN, K., B. UVNÄS and G NILSSON, Quant tativ aspects on the vagal control of gastrin release in cats. *Act physiol. scand.* 1976. *96*. 19–28

…e I. Body temperature, mean arterial blood pressure, hemoglobin concentration and acid–base …lues of arterial blood in the different groups of rats.

…roup	Temp (°C)	MABP (mmHg)	Hb (g/100 ml)	Pa_{CO_2} (mmHg)	pH	B.E. (μmol/ml)
…ocapnia …) 1015	37.1 ±0.1	133 ±7	15.1 ±0.6	41.1 ±0.7	7.371 ±0.019	0.6 ±1.1
…ocapnia …) 1015	37.0 ±0.2	137 ±8	15.3 ±0.2	14.7 ±0.4	7.638 ±0.017	0.8 ±0.7
…ercapnia …) 1015	37.1 ±0.0	129 ±3	15.1 ±0.4	82.5 ±0.9	7.178 ±0.009	4.7 ±0.6
…ercapnia hypoxia …) 1015	37.3 ±0.1	133 ±5	15.1 ±0.5	86.6 ±1.–	7.115 ±0.018	4.6 ±3.4

…es are means ±S.E. for groups of 4–6 rats.

…n by Atkinson and Walton (1967). Statistical differences were evaluated using Wilcoxon rank sum test, …pt for the monoamine data in Table IV where one-way analysis of variance followed by t-test was used …er, 1962).

Results

…siological variables

…ble I shows body temperature, mean arterial blood pressure (MABP), hemoglobin con…tration (Hb), arterial P_{CO_2} and pH, and the calculated base excess concentration (BE). …dy temperature was close t 37°C in all groups. Every animal had mean arterial blood …ssure of 120 mmHg, or higher and a hemoglobin concentration exceeding 13.2 g (100 …)$^{-1}$ In the hypocapnic group arterial P_{CO_2} was close to 15 mmHg, and in the hypercapnic …oups close to 85 mmHg. Apart from the fact that hypercapnia gave rise to a moderate …respiratory acidosis arterial plasma pH changed according to the variation in P_{CO_2}.
Table II illustrates the arterial and cerebral venous P_{O_2} values. The arterial P_{O_2} was close …100 mmHg except in the second hypercapnic group in which it was deliberately reduced. …hypocapnia and (normoxic) hypercapnia there was a highly significant decrease and in-

…BLE II. Oxygen tension of arterial as well as cerebral venous blood of rats in the different groups.

…p group	Pa_{O_2}	Pv_{O_2}
…ormocapnia …SD 1015	104 ±4	39.1 ±1.0
…ypocapnia …SD 1015	101 ±6	18.5 ±2.1
…ypercapnia …SD 1015	98 ±2	60.0 ±1.7
…ypercapnia hypoxia …SD 1013	47.6 ±2.5	38.3 ±1.5

…E. (mmHg) for groups of 4–6 animals.

HCl (NSD 1015) The experiments had the dual objective of studying the influence of changes in tissue P_{O_2} and in intracellular pH With the degree of hypocapnia induced (arterial P_{CO_2} about 15 mmHg) there is a reduction in venous (and tissue) P_{O_2} but, due to a stimulation of anaerobic glycolysis, changes in intracellular pH (pH_i) are either small or absent (see Mac Millan and Siesjö 1973) Hypercapnia induced under normoxic condition causes 2 main changes (1) a fall in pH_i and (2) an increase in venous (and tissue) P_{O_2} due to a concomitant rise in cerebral blood flow In order to separate the effects of hyperoxia and acidosis a second hypercapnic group was studied in which venous P_{O_2} was reduced to normal by the administration of a gas mixture with low oxygen content

Methods

Operative and sampling technique

Male Wistar rats, weighing 270–370 g, were used for the experiments. Anesthesia was induced ith 2 halothane. The rats were then immobilized with t bocurarine chloride, administered *i p* quickly tracheotomized and ventilated with Starling type respirator with an inflow of 70 N_2O and 30 O_2. The right femoral artery was cannulated for electromanometric blood pressure measurements and 100-μl samples of blood were drawn for nalysis of P_{O_2}, P_{CO_2}, pH and hemoglobin content A skin incision was made over the skull bone, and a burr hole was placed over the superior sagittal si us for sampling of cerebral enous blood Two samples of blood from the femoral artery were drawn for measurements of pH, P_{CO_2} and P_{O_2} It was controlled that a P_{CO_2} constancy of better than 10 between the two samples was t hand The rectal temperature was kept close to 37°C during the expt 4 groups of animals were used

Group I Normocapnia The respirator was adjusted to give arterial CO tensions of about 35–40 mmHg and arterial O tensions of about 100 mmHg. 30 min before the end of the expt NSD 1015 was administered i p. (100 mg kg⁻¹). 15 and 29 min after the administration of NSD 1015, P_{O_2}, P_{CO_2} and pH ere measured n arterial and cerebral enous blood. Thirty min after the administration of NSD 1015 liquid nitrogen was poured int plastic funnel fitted nto the ski incision over the skull bone thereby freezing the brain *in situ*. The frozen brain was split into two parts. Cortical tissue was taken from the right half of the brain for subsequent enzymatic, fluorometric analyses of glucose, glucose-6-phosphate, lactate, pyruv te, citrate, α-ketoglut rate malate ammonia glutamate, glutamine, aspartate, phosphocreatine, ATP ADP and AMP The left hemisphere was analysed for tryptophan (TRY), 5-hydroxytryptopha (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydro yindoleacetic acid (5-HIAA) tyrosine (TYR), d hydro yphenylalanine (DOPA), dopamine (DA) and noradrenaline (NA).

Group II Hypocapnia. 5 mi before the administration of NSD 1015, hyperventilation as educed to give arterial CO tensions of about 15 mmHg The animals were otherwise treated as ere the animals group I

Group III Hypercapnia. 5 min before the administrat on of NSD 1015 CO_2 was dded to the inspired gas Hyperventilation was induced. N_2O was withdraw and the f nal composition of the inspired gas mixture was about 8 % CO_2 and 25 % O_2 i N The arterial CO_2 tensions this group varied between 80 and 85 mmHg. With the exception of the induced hypercapnia the animals were treated as group II

Group IV Hypercapnia associated with hypoxia I this group the nspired gas m ture consisted of CO_2, O_2 and N I order to get arterial O tensions of bout 50 mmHg and no reduction cerebral enous O tensions, compared with normal animals, the O_2 content of the nspired gas mi ture wa reduced to about 15 % Otherwise the group was identical to group III

Analytical techniques. P_{O_2}, P_{CO_2} and pH were measured t 37°C w th microelectrodes (Eschweiler and Co Kiel Radiometer Copenhagen). Hemoglobin concentratio was measured photometrically (Vitatron) For measurements of organic phosphates, carbohydrate metabolites, ammonia, nd mino acids, the tissue was extracted at −2 °C and enzymatic, fluorometric techniques were used (f details, see Folbergrová *et al.* 1972 and b, 1974) The methods f r tyrosine, tryptophan, monoam nes nd 5-HIAA have been described previously (see Davis and Carlsson 1973 b).

Calculations. The blood base excess values were derived from the P_{CO_2}, the pH and the hemoglobin concentration according to the equation given by Siggaard Andersen (1966). The energy stat of the bra tissue was evaluated from the energy charge of the adenine nucleotide system (EC), calculated by the formula

TABLE IV Contents of tryptophan (TRY), 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydroxyindole-acetic acid (5-HIAA), tyrosine (TYR), dihydroxyphenylalanine (DOPA), dopamine (DA), and noradrenaline (NA) in the left part of the brain of rats in different groups.

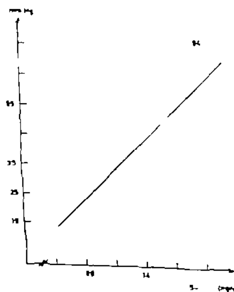
Exp group	TRY	5-HTP	5-HT	5-HIAA	TYR	DOPA	DA	NA
[illegible]	6.8	142	298	217	16.7	236	1 179	47
LSD 0.05	±0.3	±16	±31	±11	±0.7	± 4	±113	±16
[illegible]	7.2	83	333	195	14.1	196	1 27?	290
LSD 0.05	±0.4	±4	±22	±7	±0.8	±13	±125	±27
[illegible]	7.4	177	360	247	14.9	296	1 185	274
0.05	+0.4	±13	±40	±21	±0.7	±14	±60	±27
[illegible]	7.8	148	292	16	14.5	341	1 196	193
0.05	±0.6	±7	± 4	±15	±1.4	±1	±171	±27

... are means ± S.E. (μg/g wet wt for TRY and TYR, otherwise ng/g wet wt) for groups of 6-7 rats. ... from the normocapnic group: *$p < 0.05$ **p 0.001

...ous P_{O_2} and tyrosine hydroxylation. However when venous P_{O_2} was normalized under ...ercapnic conditions DOPA accumulation did not decrease. Thus, hydroxylation of ...sine does not seem to be a simple function of venous P_{O_2} but must be influenced by pH ...d (see Discussion).

Discussion

...ore discussing the results on monoamine metabolism it seems warranted to recall some ...he changes induced in the tissue by hypocapnia and hypercapnia. Since cerebral blood ...w varies directly with the CO_2 tension, and since variations in the CO_2 tension between 15 ...d 85 mmHg do not lead to changes in the cerebral oxygen uptake (for data on rats, see



... 1 Relation between oxygen tension in ...ebral venous blood and contents of 5-HTP ...brain tissue of rats. The filled circles denote ...ean values in the different groups of ani...als ... correlation coefficient.

TABLE III. Contents of phosphocreatine (PCr), creatine (Cr), ATP ADP and AMP as well as adenylate energy charge (EC) in the different groups of rats.

Exp. group	PCr	Cr	ATP	ADP	AMP	EC
Normocapnia NSD 1015	4.58 ±0 10	5.58 ±0 03	3.05 ±0 02	0.277 ±0.005	0 023 ±0 001	0.952 ±0.001
Hypocapnia NSD 1015	4 78 ±0 13	5 74 ±0.03	3 14 ±0.01	0.302 ±0 003	0 024 ±0 001	0.950 ±0.001
Hypercapnia NSD 1015	4 38 ±0 03	5 98 ±0.04	3 15 ±0 04	0.274 ±0.002	0 022 ±0.001	0.954 ±0.000
Hypercapnia + hypoxia NSD 1015	4.28 ±0.11	6.14 ±0.09	3 13 ±0 02	0.280 ±0 002	0.021 ±0.001	0.953 ±0 001

Values are means ±S.E. (μmol/g wet wt) for groups of 4–6 rats.
Differs from the normocapnia group $p<0\ 05$ $p<0\ 02$.

crease in venous P_{O_2}, respectively In the hypoxic hypercapnic group the venous P_{O_2} was close to normal

Organic phosphates carbohydrate metabolites ammonia and amino acids

These measurements were carried out to secure that the administration of NSD 1015 did not by itself influence the metabolic state of the tissue. Table III gives the values for PCr creatine, ATP ADP AMP and adenylate energy charge. The normocapnic group given NSD 1015 had values for these labile substances that were very close to those previously obtained in the laboratory (*cf* Folbergrová *et al* 1972 a, MacMillan and Siesjö 1973), and we conclude that NSD 1015 does not influence cerebral energy state (*cf* Davis *et al* 1973). There was a small rise in ATP content in the hypocapnic and hypercapnic groups but the adenylate energy charge did not differ between the groups. In the hypocapnic group, there was a small increase in ADP but this increase can be observed also in animals who have not received NSD 1015 (MacMillan and Siesjö 1973) We conclude that all groups studied had an essentially normal energy state and that the presence of NSD 1015 did not influence energy balance during the hypocapnic and hypercapnic conditions.

Analysis of tissue concentrations of glucose, glucose-6-phosphate, pyruvate, lactate citrate, α-ketoglutarate, malate, glutamate, glutamine, aspartate and ammonia showed that NSD 1015 had no significant effect on carbohydrate metabolites, amino acids or ammonia under normocapnic, hypocapnic or hypercapnic conditions.

Monoamine metabolism

The values are given in Table IV Since all other variables remained essentially constant in the various groups, attention should be directed towards changes in the concentrations of 5-HTP and DOPA 5-HTP and DOPA concentrations were decreased in hypocapnia and increased in hypercapnia. Statistical significance was, however not reached for the slight DOPA induced decrease in hypocapnia. 5-HTP formation was normalized when P_O was lowered during hypercapnia. Thus, hydroxylation of tryptophan seems to vary directly with the venous P_{O_2} (Fig. 1) Under normoxic conditions a similar relationshi[illegible]

TABLE IV. Contents of tryptophan (TRY), 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydroxyindole-acetic acid (5-HIAA), tyrosine (TYR), dihydroxyphenylalanine (DOPA), dopamine (DA), and noradrenaline (NA) in the left part of the brain of rat in different groups.

group	TRY	5-HTP	5-HT	5-HIAA	TYR	DOPA	DA	NA
normocapnia	6.8	142	298	17	16.7	36	1 179	47
SD 1015	±0 3	±16	±31	±11	±0.7	± 4	±113	±16
hypocapnia	7 2	83	333	193	14 1	196	1 277	780
SD 1015	±0 4	±4	±22	±7	±0 8	±13	±125	± 77
hypercapnia	7 4	177	360	247	14 9	296	1 185	274
SD 1015	_0 4	±13	±40	±21	±0.7	±14	_ 64	_27
hypercapnia + hypoxia	7 8	148	292	216	14.5	341	1 196	193
SD 1015	_0 6	±7	±24	±15	±1 4	±12	±171	±77

Values are means ±S.E. (µg/g wet wt for TRY and TYR, otherwise ng/g wet wt) for groups of 6–7 rats. Differs from the normocapnia group. *p 0 05 **p 0 001

between P_{O_2} and tyrosine hydroxylation. However when venous P_{O_2} was normalized under hypercapnic conditions DOPA accumulation did not decrease. Thus, hydroxylation of tyrosine does not seem to be a simple function of venous P_{O_2} but must be influenced by pH as well (see Discussion).

Discussion

Before discussing the results on monoamine metabolism it seems warranted to recall some of the changes induced in the tissue by hypocapnia and hypercapnia. Since cerebral blood flow varies directly with the CO tension, and since variations in the CO tension between 15 and 85 mmHg do not lead to changes in the cerebral oxygen uptake (for data on rats, see

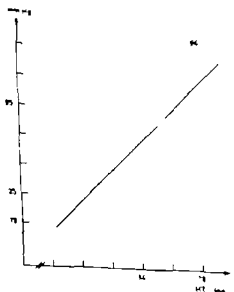


Fig 1 Relation between oxygen tension in cerebral venous blood and contents of 5-HTP in brain tissue of rats. The filled circles denote ...ment values in the different groups of animals. correlation coefficient.

Eklöf *et al* 1973) It follows that hypocapnia is accompanied by decreased and hypercapnia by increased P_{O_2} in brain tissue. In view of the results reported by Davis and Carlsson (1973 a and b see also Davis *et al* 1973) it would be expected that hypocapnia should decrease and hypercapnia increase the rate of hydroxylation of tyrosine and tryptophan. However *this is so only if the accompanying acid–base changes do not influence the hydroxylation* Hypercapnia is invariably associated with a decrease in intra and extracellular pH of the brain (see Siesjö *et al* 1972) In order to differentiate between effects due to hyperoxia and acidosis arterial P_{O_2} was reduced in one hypercapnic group so as to normalize cerebral venous P_{O_2}. A decrease in P_{CO_2} *i.e* hypocapnia, increases intra and extracellular pH However when Pa_{CO_2} is reduced to about *15* mmHg there seems to be sufficient stimulation of anaerobic glycolysis to achieve normalization of pH_i (MacMillan and Siesjö 1973). Thus in the present hypocapnic group there should have been a decrease in tissue P_O but no, or only a very small change in pH_i

In the present experiments, the tissue concentrations of tryptophan 5-HTP 5-HT 5-HIAA, tyrosine, DOPA, dopamine and noradrenaline were measured in animals given NSD 1015 The results suggest that the hydroxylation of tryptophan, *i.e* the first step in the synthesis of 5-HT varies directly with the tissue P_{O_2} and that pH changes have little influence. In contrast the hydroxylation of tyrosine did not show a simple relationship to P_{O_2}. The results suggest that pH changes contribute to the increased rate of synthesis of DOPA under hypercapnic conditions. That a difference in response to pH changes exists between the hydroxylation of tryptophan and of tyrosine is also supported by the data of Brown *et al* (1974 see Introduction)

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References

ATKINSON, D E and G M WALTON, Adenosine triphosphate conservation in metabolic regulation. *J biol Chem.* 1967 *242*. 3239–3241

BROWN R M S. R. SNIDER and A. CARLSSON, Changes in biogenic amine synthesis and turnover induced by hypoxia and/or foot shock stress. II The central nervous system. *J Neural Transm* ... 1974 *35* 293–305

DAVIS, J N and A CARLSSON, Effect of hypoxia on tyrosine and tryptophan hydroxylation in unanaesthetized rat brain. *J Neurochem.* 1973 *20* 913–915

DAVIS, J N and A. CARLSSON, The effect of hypoxia on monoamine synthesis, levels and metabolism in rat brain. *J Neurochem.* 1973 b. *21* 783–790.

DAVIS, J N A. CARLSSON, V MACMILLAN and B K SIESJÖ, Brain tryptophan hydroxylation Dependence on arterial oxygen tension. *Science* 1973 *182*. 72 74

EKLÖF B N A. LASSEN, L. NILSSON, K. NORBERG and B. K. SIESJÖ, Blood flow and metabolic rate for oxygen in the cerebral cortex of the rat *Acta physiol scand.* 1973 *88* 587 589

FISHER, D B. and S. KAUFMAN, The inhibition of phenylalanine and tyrosine hydroxylases by high oxygen levels. *J Neurochem.* 1972 *19* 1359–1365.

FOLBERGROVÁ, J V MACMILLAN and B. K. SIESJÖ, The effect of moderate and marked hypercapnia upon the energy state and upon the cytoplasmic NADH/NAD ratio of the rat brain. *J Neurochem.* 1972 a. *19* 2497 2505.

[illegible], L., V. MacMillan and B. K. Siesjö, The effect of hypercapnic acidosis upon some glycolytic and Krebs cycle-associated intermediates in the rat brain. *J. Neurochem.* 197? b. *19*, 2507–2517.

[illegible], J., U. Ponten and B. K. Siesjö, Patterns of changes in brain carbohydrate metabolites, amino acids and organic phosphates at increased carbon dioxide tensions. *J. Neurochem.* 1974, *2?*, 1115–1125.

[illegible], H. and J. L. Sawyer, Demonstration, characterization, and assay procedure of tryptophan hydroxylase in rat brain. *Analyt. Biochem.* 1966, *15*, 53–64.

[illegible]cMillan, V. and B. K. Siesjö, Brain energy metabolism in hypoxemia. *Scand. J. clin. Lab. Invest.* 1972, *3?*, 127–136.

[illegible]cMillan, V. and B. K. Siesjö, The influence of hypocapnia upon intracellular pH and upon some carbohydrate substrates, amino acids and organic phosphates in the brain. *J. Neurochem.* 1973, *21*, [illegible]3–1299.

[illegible], K. and B. K. Siesjö, Cerebral metabolism in hypoxic hypoxia. I. Pattern of activation of glycolysis, [illegible] re-evaluation. *Brain Res.* 1975, *86*, 31–44.

[illegible], B. K. and L. Nilsson, The influence of arterial hypoxemia upon labile phosphates and upon extracellular and intracellular lactate and pyruvate concentrations in the rat brain. *Scand. J. clin. Lab. Invest.* [illegible]971, *27*, 83–96.

[illegible], B. K., J. Folbergrová and V. MacMillan, The effect of hypercapnia upon intracellular pH in the brain, evaluated by the bicarbonate-carbonic acid method and from the creatine phosphokinase equilibrium. *J. Neurochem.* 1972, *19*, 2483–2495.

[illegible]-Andersen, O. Titratable acid or base of body fluids. *Ann. N. Y. Acad. Sci.* 1966, *133*, 41–58.

[illegible], B. J. *Statistical Principles in Experimental Design.* McGraw Hill, New York, 196[illegible].

References

EMÅS, S and B FYRÖ, Gastrin-like activity in different parts of the gastrointestinal tract of the cat. *Acta physiol. scand* 1968 74 359–367

NILSSON, G Increased plasma gastrin levels in connection with inhibition of gastric acid responses to sham feeding following bulbar perfusion with acid in dogs *Scand. J Gastroent* 1975 10 273–278

NILSSON, G R S. YALOW and S. A. BERSON, Distribution of gastrin in the gastrointestinal tract of human, dog, cat and hog. *Nobelsymposium XVI* Almqvist & Wiksell, Stockholm, 1973 223–238.

OLBE, L., Effect of resection of gastrin releasing regions on acid response to sham feeding and insulin hypoglycemia in Pavlov pouch dogs. *Acta physiol. scand* 1964 62 169–175.

SJÖDIN, L., Potentiation of the gastric secretory response to sham feeding in dogs by infusions of gastrin and pentagastrin. *Acta physiol scand.* 1972 85 4–32

SJÖDIN L. and G NILSSON Role of antrum and duodenum in the control of postprandial gastric acid secretion and plasma gastrin concentration in dogs. *Gastroenterology* 1975 69 928–934.

THOMPSON, J C., D D REEDER, W D DAVIDSON, B. M. JACKSON and B. G CLENDINNEN, Studies on the metabolism of gastrin *Nobelsymposium XVI* Almqvist & Wiksell, Stockholm 1973 111 135.

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SI units with recommended symbols

Units	*Symbols*
kilogramme	kg
second millisecond	s ms
mole, millimole micromole nanomole picomole	mol mmol μmol nmol pmol
meter millimeter micrometer nanometer	m mm μm nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s^{-1})
newton (force)	N (kg m/s^2)
pascal (pressure)	Pa (N/m^2)
joule (energy)	J (N m)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd sr)
lux (illumination)	lx (lm/m^2)

Conversion factors to be given in Methods.

Permitted non-SI units

Units	*Symbols*
gramme	g
minute	min
hour	h
molarity (mol/liter)	M
(calorie)	cal (4 184 J)*
(kilopond)	kp (9 81 N)
(millimeters of mercury)	mm Hg (1.333 bar)*
(millibar)	mbar (100 Pa)*
curie	Ci
liter milliliter microliter	l ml μl
degree Celsius	°C